OL

Perspectives in supramolecular chemistry: self-assembly in solution

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Molecular chemistry has developed a wide range of very powerful procedures for building ever more complicated molecules and materials. Supramolecular chemistry aims at constructing highly complex chemical systems and advanced materials by designing arrays of components held together by intermolecular forces.

The concepts of supramolecular chemistry have been based on and expressed in the oligomolecular supermolecules. Their extension to polymolecular entity leads into the area of supramolecular assemblies and organised phases. In particular, one may envisage the implementation of *molecular recognition* as a means for controlling the evolution and the architecture of polymolecular species as they spontaneously build up from their components through *self-organisation*. Such designed recognition directed self-assembly is of major interest in supramolecular design and engineering.

Molecular information expressed through molecular recognition events provides means for directing the spontaneous formation of supramolecular species from complementary components.

Various approaches to self-assembling systems of either organic or inorganic nature have been pursued. Several of them will be described.

General reference

J.-M. Lehn (1995) Supramolecular chemistry: concepts and perspectives. VCH, Weinheim

CL

Structure and function of large proteins and protein assemblies by x-ray crystallography and electron microscopy

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By electron microscopy the size and shape of large proteins can be visualized. Subunits of aggregates may be identified by the generation and analysis of defined fragments or by specific labelling. This information combined with high resolution crystal structure determination of the subunits allows a reliable reconstruction of the large assemblies in atomic detail. When well-ordered two-dimensional preparations are available, substantially improved resolution can be obtained by electron microscopy and molecular details recognized. A direct comparison is then possible with crystal structures and structural differences may be defined. Decoration of crystals with heavy metals and analysis by electron microscopy allows identification of molecules in crystal lattices and determination of their orientation and location as a starting point for the x-ray crystallographic analysis. Examples are given:

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PL-1

SINGLE MOLECULE FORCE SPECTROSCOPY BY AFM

M.Rief, M.Gautel, J. Fernandez, P. Oesterhelt, H.Li, H.E. Gaub

Recent developments in piconewton instrumentation allow the manipulation of single molecules and measurements of intermolecular as well as intramolecular forces. We took advantage of the high spatial resolution of the AFM and performed mechanical experiments with individual polymers and proteins. Dextran filaments anchored on a gold surface were picked up with the AFM tip and stretched. The elongation of individual molecules was recorded as a function of the applied load. We found that at low forces the deformation of dextran is dominated by entropic forces and is well described by the Langevin function with a Kuhn length of 6 Å. At elevated forces the dextran filaments show an additionalsegment elasticity of 650 pN/Å.which is attributed to the deformation of the bond angles. At a force of 300 pN the dextran filaments undergo a massive conformational change and give by 0.6 Å per ring. The high force conformation exhibited a segment elasticity of 1700 pN/Å. The conformational change was found to be reversible and was corroborated by molecular force field calculations. In a similar fashion, Titin, a large muscle protein was investigated. The elongation of individual native molecules was measured as a function of the applied load for different rates. A massive conformational change was found to occur at forces between 150 and 250 pN. Measurements with several recombinant Titin Ig-segments of different length allowed us to identify this conformational change as the unfolding of individual Ig-domains. The refolding was found to be only partial and strongly rate dependent.

PL-2

SEQUENCE-SPECIFIC RECOGNITION OF DNA AND SELECTIVE CONTROL OF GENE EXPRESSION.

C. Hélène[+], T. Garestier[+], J.S. Sun[+], U. Asseline[*], N.T. Thuong[*]

Specific sequences of the DNA double helix can be recognized by oligonucleotides forming a local triple helix at oligopurine-oligopyrimidine sequences (1). Introduction of N3'→ P5' phosphoramidate linkages instead of phosphodiesters leads to much more stable triple-helical complexes (2). Covalent attachment of an intercalating agent at either end of the oligonucleotide provides additional stability (3). The discovery of triple helix-specific intercalating agents (4) opens the possibility of designing novel oligonucleotide-intercalator conjugates that exhibit higher binding and selectivity for double-helical DNA (5). Triple helix formation by oligonucleotide-intercalator conjugates has opened a new field of investigation aimed at controlling gene expression at the transcriptional level (2,3). Taken together with the demonstration that DNA sequences are accessible within the chromatin structure of cell nuclei (6) these results open the possibility of developing new therapeutic strategies targeting specific genes in pathological disorders.

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PL-3

DNA STRUCTURE AND RECOGNITION,

Abdelali Kettani, Serge Bouaziz, Chin Lin and Dinshaw J. Patel.

The lecture will cover two topics of current interest in DNA structure and recognition in our laboratory. We use NMR and molecular dynamics calculations to determine unusual DNA structures and their complexes in solution.

Solution structures will be reported on DNA quadruplexes containing base tetrads and triads. The emphasis will be on pairing alignments other than standard G-tetrads within the quadruplexes. We shall also provide molecular explanations for the monovalent cation dependence of conformational equilibria in DNA quadruplexes.

Solution structures will be reported on DNA aptamers complexed to amino acids and mononucleotides. The focus will be on the principles, patterns and diversity of DNA folding and ligand recognition in these systems. These ligand-DNA aptamer complexes will be compared with their ligand-RNA aptamer counterparts.

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PL-4

THE ROTARY MECHANISM OF ATP SYNTHESIS

John E. Walker

In the atomic structure of F_1-ATPase from mitochondria, the three catalytic β-subunits have different conformations and different occupancies of nucleotides. The ATP analogue, AMP-PNP, is bound to one β-subunit, ADP to the second, and no nucleotide is bound to the third. The structure is unchanged by the binding of the inhibitors aurovertin and efrapeptin, and their binding sites are consistent with earlier evidence about their locations in active F_1-ATPase. Therefore, the determined structure of F_1-ATPase probably represents a state in the active catalytic cycle. It also suggests that during catalysis the three conformations of β-subunits are interconverted by the relative rotation of a central anti-parallel α-helical coiled-coil structure in the γ-subunit, around which the three β-subunits and the three non-catalytic α-subunits are arranged alternately. So, the structure supports a binding change mechanism which proposed a cyclic inter-conversion of three different catalytic sites during the enzyme's catalytic cycle. There is now clear evidence of rotation of the γ-subunit during ATP hydrolysis.

How can a rotary motion be generated by the transport of protons through F_O, and how is the energy for ATP synthesis carried from F_O to the catalytic sites about 100 Å away in F_1? The proteins linking F_1 and F_O must be involved in these processes. They are organized in two separate domains. In the simplest bacterial F_1F_O-ATPases, a central stalk seems to consist of part of the γ-subunit, which interacts with subunit c in F_O and the ε-subunit, which binds to both the γ- and β-subunits. The two identical b subunits and the δ-subunit may make a separate peripheral structure in which the δ-subunit interacts with the N-terminal part of the α-subunit, in the membrane-distal part of F_1. The mitochondrial enzyme has a similar central stalk made of subunits γ, δ and ε, and a peripheral structure made of one copy each of subunits b, d, OSCP and F_6. The central structure is probably involved directly in energy transmission, and the peripheral feature could serve as a stator to counter the tendency of $\alpha_3\beta_3$ to rotate in response to a rotating γ-subunit.

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1-1

NMR OF PARAMAGNETIC METALLOPROTEINS. I. Bertini
It will be shown how it is possible : 1) to optimize the NMR spectra in some paramagnetic systems, 2) to include nuclear relaxation as structural constraints, and 3) to include hyperfine shifts as structural constraints.
All of these data provide well resolved solution structures. Examples will be given of Fe-S proteins and cytochrome c.

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1-2

C.I. Branden.

No abstract available.

1-3

STRUCTURAL STUDIES OF EPITOPES IN PICORNAVIRUSES.
N. Verdaguer [1], E. Hewat [2], M.G. Mateu [3], E. Domingo [3], D. Blaas [4], I. Fita [1].
The antigenic properties of picornaviruses have been extensively studied but our understanding of the molecular mechanisms of viral neutralisation by antibodies is still limited. A better knowledge of virus/antibody structures for a range of antibodies with different neutralizing properties might be clarifying. However, crystals diffracting at high resolution of virus-antibodies complexes are extremely difficult to obtain. Cryo-electron microscopy and 3-D reconstruction of images of those complexes have already demonstrated their value in this field. When combined with X-ray crystallographic data of the constituent elements a more complete picture of the virus-antibody interactions can be obtained.
We report crystallographic structural studies of Fab fragments from neutralizing antibodies rised against foot-and-mouth disease virus serotype C (FMDV-C) and human rhinovirus serotype II (HRV2) complemented with model building analysis and cryo-electron microscopy data and 3D reconstruction from complexes of the intact viruses with the antibodies or with saturating concentrations of FBA fragments.
For FMDV the structure of the immuno-dominant site A has been determined and the direct implication of the receptor recognition RGD motif in the interaction with the antibodies has been observed. FMDV and HRV are particularly relevant representatives of the highly variable RNA viruses and FMDV it is the economically most important animal pathogen world-wide. The knowledge gained in these neutralization structural studies have a number of implications that could be helpful for virus control.

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1-4

INTERACTION STUDIES OF THE TRANSCRIPTION FACTOR ALCR WITH ITS DNA TARGET BY NMR : METHODOLOGICAL ASPECTS AND RESULTS. R. Cerdan, C. Van Heijenoort , N. Birlirakis, B. Cahuzac, G. Goussard, B. Felenbok and E. Guittet.
AlcR is a transcription factor from *Aspergillus nidulans* required for the transcriptional activation of the genes encoding the ethanol metabolizing enzymes. It belongs to a family of activators containing a Zn_2Cys_6 motif, whose most representative member is GAL4.
The three-dimensional structure of some complexes of transcription factors containing Zn-binuclear clusters with their target DNAs (GAL4, PPR1...) have been recently determined by X-ray cristallography and by NMR. They all show direct contacts of the protein with a short consensus half-site and demonstrate, in addition to biological evidences, that the selectivity for the complete site is due to protein-protein interactions.
AlcR is shown by NMR to form stable complexes with oligonucleotide sequences comprising the consensus half-site 5'-TGCGG-3'. AlcR is thus a unique exemple of a Zinc cluster able to strongly interact with a half-site. This could explain why both palindromic and direct repeats are recognized by the protein. Extensive NMR studies have been performed on the protein and on its complex with a half-site. Analysis of the protection factors of the exchangeable protons by new NMR approaches, evaluation of the variation of the dynamics of the protein upon binding and observation of intermolecular NOEs between the protein and the DNA, provide an accurate model for the interaction. It demonstrates much more extensive contacts between the two partners than expected.

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1-5

LIGAND BINDING AND PROTEIN DYNAMICS. G. U. Nienhaus, J. D. Müller, B. H. McMahon, H. Frauenfelder, J. B. Johnson and D. C. Lamb.

Proteins can assume a large number of conformational substates that endow them with flexibility. Often, a few discrete 'taxonomic substates' can be distinguished. They are of particular interest because they can be characterized individually, and they are therefore amenable to detailed structure-function studies. In carbonmonoxy myoglobin, the multiple infrared absorption bands of the CO bound to the heme iron have been assigned to a small number of taxonomic *A* substates. Their relative populations depend on environmental factors, such as temperature, pressure, and pH.

We have determined the kinetics of conformational transitions between the *A* substates between 170 and 300 K using flash photolysis with infrared monitoring and pressure relaxation experiments. The temperature dependence of the rates implies that the *A* substate interconversions involve collective motions of larger domains. Systematic studies of the pH dependence of the infrared spectra link some of the *A* substates to protonations of specific side chains, whereas others are clearly independent of pH. Using mutant myoglobins, we were able to show that the pH dependence of the *A* substate spectra is governed by two residues near the active site, His64 and His97. Furthermore, the pH dependence of the CO association rates at room temperature was measured and modeled with a superposition of *A* substates with different rebinding properties. Consequently, the ligand binding properties of myoglobin can be controlled by changing the relative populations of the *A* substates.

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1-6

SPATIAL DESCRIPTION OF THE CHARGE DISPLACEMENT IN BACTERIORHODOPSIN DURING FUNCTION, P. Ormos, A. Dér

The function of proteins, especially that of active ion transporters, is accompanied by charge displacements. This charge displacements contain crucial information about the details of the important conformational transitions. A number of photoelectric methods have been developed to follow the charge displacements, all based on model systems where the proteins are arranged in an asymmetric fashion. However, all previously applied methods use model systems of cylindrical symmetry and therefore only one component of the displacement can be detected, typically the one pointing in the direction of the ion transport.

We have developed a novel method that applies photoselection with special geometry by which the photoelectric currents within the light driven proton pump Bacteriorhodopsin can be separately followed in all three spatial directions. Thus a complete characterization of the charge displacements following light excitation is possible. Based on this method the transitions during the proton pumping process can be determined. Consequently, it is possible to identify the molecular model (from the numerous existing theoretical suggestions) to describe the functional protein conformational transitions. This approach gives fundamental progress in the understanding of the steps of biological energy transduction.

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1-7

DYNAMICS-HYDRATION-FUNCTION RELATIONSHIPS IN PURPLE MEMBRANES : NEUTRON STUDIES. G. Zaccai.

Purple Membranes (PM) from *Halobacterium salinarium* contain one protein, bacteriorhodopsin (BR), organised with lipids on a highly ordered two-dimensional lattice. BR binds a molecule of retinal and functions as a light driven proton pump. Its structure has been solved by electron microscopy to relatively high resolution for a membrane protein *in-situ* [Henderson et al. (1990)*J. Mol. Biol.* **213**, 899-929]. Because of their remarkable crystallinity, PM present an exceptional opportunity to describe structural and dynamics relationships between the lipid, water and protein membrane components. Neutron diffraction experiments on PM have allowed to define hydration in the membranes under different conditions. Following a hypothesis relating BR function to hydration and dynamics [Zaccai (1987)*J. Mol. Biol.* **194**, 569-572] neutron spectroscopy experiments have characterised the amplitudes of thermal motions in PM as a function of temperature. Experiments were performed at the Institut Laue Langevin in Grenoble on unlabelled PM where the mean motions of all atoms were measured [Ferrand et al. (1993) *Proc. Natl. Acad. Sci. (USA).* **90**, 9668-9672] and on H-D labelled material with a focus on the motions of the retinal binding pocket and the extracellular half of BR [Réat et al., submitted]. In support of the hypothesis, function, dynamics and hydration are strongly correlated. Furthermore, the protein appears to have a "harder" core surrounded by "soft" regions thus reconciling functional flexibility for transfer along the proton pathway and the "valve" requirement associated with vectorial transport.

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1-8

THE DYNAMICS OF MYOGLOBIN INVESTIGATED BY RAYLEIGH SCATTERING OF MÖSSBAUER RADIATION (RSMR) USING ^{183}W
K. Achterhold, C. Keppler, F. Parak

Protein molecules have a well defined but nevertheless flexible structure. Both features are essential for their functions. We describe an experiment where the average dynamic properties of myoglobin are investigated by scattering of the ^{183}W Mössbauer radiation with $\lambda = 0.268$ Å. In this RSMR experiment γ- quanta are Rayleigh scattered by the sample under investigation and the energy of the scattered quanta is analysed with the help of the Mössbauer effect. While in RSMR experiments using ^{57}Fe as Mössbauer source ([1],[2]) the dynamics of myoglobin is probed on a timescale faster than 100ns, the use of ^{183}W measures motions on a timescale faster than 0.2ns. This timescale is also accessible with computer simulations and incoherent neutron scattering. In contrast to neutron scattering experiments, which reveal the motions of the hydrogens of the molecule, the RSMR technique measures mainly the dynamics of the heavier atoms (C,N,O). Moreover, the RSMR technique using ^{183}W overcomes problems connected with the usually low counting rates. First experiments have shown that the lineshape of the scattered radiation depends on the scattering angle indicating diffusive motions in limited space. [1] Krupyanskii YuF, Parak F, Goldanskii VI, Mössbauer RL, Gaubmann EE, Engelmann H, Suzdalev IP (1982), Z. Naturforsch. **37c**: 57-62; [2] Krupyanskii YuF, Goldanskii VI, Nienhaus GU, Parak F (1990), Hyp. Int. **53**: 59-74.

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1-9

DESTABILISATION OF CALMODULIN N- & C-DOMAINS BY POINT MUTATIONS. J.P.Browne, M.Strom, S.R.Martin, and P.M.Bayley.

Calmodulin (CaM) binds 4 Ca ions with high affinity, and the resulting complex shows high thermal stability with T_m > 100 °C. We have made single residue mutations of the hydrophobic Ile or Val residue in position 8 of the Ca-binding loops in sites I-IV of *Drosophila* CaM. This residue, which is part of the hydrophobic core of either CaM domain, is involved in the structural link of two Ca binding sites via a short anti-parallel β-sheet. In apo-CaM, replacement of Ile (or Val) by Gly causes extensive unfolding of the mutated domain as shown by near- and far-UV CD and fluorescence spectroscopy. At 25°C this corresponds to half of the domain's α-helical structure. On binding Ca ion, this loss in α-helix is restored for the mutants at sites I-III, but not at site IV, which requires the further binding of a high affinity target sequence to restore the native structure. The extent of the destabilisation is seen in the depression of the T_m of individual domains; this can be ~ 80 degrees eg. for Ca_4-CaM(V136G). Nevertheless, the affinity of these mutants for Ca, and the affinity for a target peptide from skeletal muscle myosin light chain kinase, are only slightly reduced compared to the wild-type. Further, the secondary structure is largely preserved in the presence of 15% v/v TFE. These results show that the domain stability of CaM can be dramatically reduced by a single mutation of a highly conserved residue, but that the binding to a target sequence can readily compensate for this, and maintain the wild-type properties. Thermodynamic analysis suggests that the unusual sensitivity of either domain to this mutation involves the disruption of hydrophobic interactions. The small domain size (~75 residues), and high transition temperatures suggest relatively low unfolding entropy and enthalpy for the native CaM structure, particularly in the apo-form.

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1-10

FOLDING PROPENSITY OF ISOLATED ANNEXIN DOMAINS: AN NMR CONFORMATIONAL STUDY. F. Cordier-Ochsenbein, R. Guerois, F. Russo-Marie, P.N. Lirsac, J.M. Neumann et A. Sanson.

Annexins constitute an interesting model for folding studies on multidomain proteins, because of their highly hierarchic structure: four consecutive homologous domains assembled into two modules (domaines 2-3 and 1-4). In the native structure, each domain (~ 70 residues) exhibits the same topology, *i.e.* five helices folded into a characteristic super-helix motif: (A-loop-B)-C-(D-E). Using NMR and CD spectroscopy, we have undertaken a «dissection» of the folding propensities in the annexin I with the aim of understanding some aspects of the relation between sequence and folding pathways. Remarkably, the recombinant annexin I domain 1 constitutes an autonomous folding unit whereas annexin I domain 2 is essentially unfolded, with, from CD measurements, only 30 % of the native helix content. The annexin V domain 1 is also able to fold independently, indicating that this property could be a general feature of the annexin family. Detailed analysis of the unfolded state of the annexin I domain 2 shows the presence of both native and non-native residual local structures. Interestingly, important residues involved in the persistent non-native local structures are also involved in crucial long range interactions in the native state. This implies a switching from local, and unfolded state-stabilising, interactions to non-local, and folded state-stabilising interactions, during the late steps of the folding process. We hypothesise that the non-native local structures, as well as the native one's, actively control, structurally and kinetically, part of the folding pathways of the protein. Taken together with data on the 2-3 module, our results lead us to suggest a sequential model for the folding process of annexin I.

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1-11

PROTEIN INTERACTIONS AND CRYSTAL GROWTH

S. Finet, F. Bonneté, A. Tardieu

The ability of lysozyme to crystallize in the presence of various ions has been extensively investigated. The influence of various salts on the protein-protein interactions of undersaturated lysozyme solutions at constant pH and temperature had previously been characterized by Small Angle X-rays Scattering (SAXS-LURE). The efficiency of anions to displace the interaction potentials towards more attractive regimes was shown to be the same as that observed in inducing crystallization(1). In the present study, investigations were extended to supersaturated solutions. Second virial coefficients A2 were measured as a function of ionic strength and temperature by SAXS and osmotic pressure. As in undersaturarted solutions, the ability of the anions to decrease the values of the A2 coefficient follows the reverse order of the Hofmeister series. A combination of SAXS and gel techniques(2) was then used to follow the X-rays scattering with time. In the first series of experiments, the supersaturation, induced by lowering the temperature, was chosen to have nucleation in about half an hour. Then, crystal growth occurred. The decrease of protein concentration and the variation of the interactions were recorded (crystals could reach 300 microns in roughly 6 hours). In these conditions, no intermediate species between monomeric form and crystal could be observed during crystal growth.

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(2) *F. Bonneté, O. Vidal, M.C. Robert, A. Tardieu,* J. Crystal Growth 168 (1996) 185-191

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1-12

PICOSECOND MOLECULAR MOTIONS IN BACTERIORHODOPSIN AS PROBED BY NEUTRON SCATTERING. J. Fitter, R.E. Lechner and N.A. Dencher

The internal molecular motions of bacteriorhodopsin (BR) in the purple membrane (PM) have been studied using quasielastic incoherent neutron scattering (QINS). We performed measurements at different energy resolutions which give access to a time range from 0.1 to a few hundred picoseconds. The dynamical behaviour of the protein-lipid complex, characterized by internal diffusive reorientational and vibrational motions, have been investigated in dependence on environmental conditions (temperature, hydration and the protein-lipid ratio). At least the temperature and the hydration of the protein-lipid complex are known to be important parameters for the functionality of BR (light-driven proton pump). Key steps of the working cycle (proton release at the Schiff-base, decay of M-intermediate, conformational change of the three dimensional structure) are known to be suppressed or even vanish at low temperatures as well as at low hydration levels. These functional aspects are accompanied by drastic changes of the protein flexibility at the picosecond time scale. Below temperatures of about 180-220 K all diffusive motions vanish and only rather fast (with correlation times τ < 1 ps) vibrational motions with much smaller amplitudes remain ("dynamical transition"). The hydration level mainly affects rather slow diffusive motions (with τ > a few ps), which are strongly reduced at low hydration levels as compared to high hydration levels. With respect to these results we discuss those dynamical properties of proteins, that are supposed to be essential for their function.

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1-13

THERMODYNAMICS AND KINETICS OF THE THERMAL UNFOLDING OF PLASTOCYANIN. D. Milardi, C. La Rosa, D. Grasso, R. Guzzi, L. Sportelli, C. Fini.

The thermal denaturation of Plastocyanin in aqueous solution was investigated by means of DSC, ESR and OD experiments, with the aim of determining its thermodynamic stability and the thermally induced conformational changes of its active site. DSC and OD experiments have shown an irreversible denaturation path. Nevertheless, by the extrapolation of the optical and heat capacity data to infinite scan rate, we were able to calculate the thermodynamic and the kinetic parameters related to the denaturation steps. Both the denaturation model proposed and the parameters extracted from the calorimetric data, have been verified by means of an accurate computer simulation using an equation containing all the information necessary to fully describe the denaturation process in detail. ESR and OD measurements, allowed us to elucidate the structural changes of the copper environment in the native and denaturated states. The results show that the disruption of the active site precedes the global protein denaturation and that the geometry of the copper-ligand atoms changes from tetrahedral to square planar. The thermodynamic enthalpic changes, the half-width transition temperature, and the value of ΔCp calculated according to an approach taking into account the common features of protein unfolding and dissolution of hydrophobic compounds, were used to evaluate the thermodynamic stability, ΔG, of the reversible process over the entire temperature range of denaturation. The lower thermal stability of Plastocyanin, compared to that of Azurin, a protein of the same structural class, has been related to the structural factors stabilizing the native state of a protein.

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1-14

NANOSECOND EVENTS IN CYTOCHROME C REFOLDING MONITORED BY STEP SCAN FT- IR SPECTROSCOPY.

Ekkehard Kauffmann, Jens Linge, Heinz-Jürgen Steinhoff and Klaus Gerwert.

New methods are being used to study the refolding of cytochrome c by time resolved FT-IR spectroscopy in the time range from nanoseconds to milliseconds. Optical triggers can initiate the refolding within picoseconds to nanoseconds and allow to investigate fast events in protein folding, occuring in the dead time of rapid mixing experiments. By time resolved FT-IR spectroscopy folding of proteins can be monitored on an atomic level and elements of secondary structure be identified (1,2).

Our first approach is based on the lower stability of ferro-cytochrome c with carbonmonoxide as sixth ligand at the heme iron compared to the species with the natural ligand. Exposing cytochrome c with carbonmonoxide bound to a laser flash results in dissociation of the carbonmonixide from the iron and, at medium guanidine hydrochloride concentration, in refolding (3). Using this method, we obtained step scan FT-IR spectra with a time resolution of 30 ns. The second approach makes use of the lower stability of ferri-cytochrome c to denaturation compared to ferro-cytochrome c. At a guanidine hydrochloride concentration where ferri-cytochrome c is unfolded while ferro-cytochrome c is still folded, refolding can be triggered by transfering an elektron to ferri-cytochrome c. Employing caged electron donors, the refolding can be initiated by a laser flash within nanoseconds (4). This method is for the first time successfully combined with time resolved FT-IR spectroscopy, the results are presented and discussed.

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1-15

DYNAMICS OF MYOGLOBIN INVESTIGATED BY INELASTIC NUCLEAR AND INELASTIC RAYLEIGH SCATTERING OF SYNCHROTRON RADIATION

C.Keppler, K.Achterhold, A.Ostermann, U.van Bürck, W.Potzel, A.I.Chumakov, A.Q.R. Baron, R.Rüffer and F.Parak

X-rays of 14.413 keV energy and a 4.4 meV bandwidth were selected by a nested monochromator system from pulsed radiation (~100 ps duration every 176 ns) of an undulator at the beamline ID18 of ESRF in Grenoble. The energy was tuneable in the range of ±100 meV. The radiation was scattered by a sample of small deoxy-myoglobin crystals enriched in ^{57}Fe and detected in a time resolving avalanche photo diode. The radiation is promptly Rayleigh scattered by the electrons of the sample or resonantly absorbed by the ^{57}Fe nuclei if the incoming radiation fits the Mössbauer transition. Reemission follows with a characteristic time of τ=141 ns of ^{57}Fe. With an incoming energy difference ΔE to the Mössbauer level, nuclear absorption only happens when a phonon compensates this difference. The integrated delayed intensity gives therefore the phonon density as a function of ΔE. Phonon spectra where measured at T=73, 124, 189 and 295 K showing for instance a maximum near the iron-histidine stretching vibration measured by Raman scattering (27 meV). Whereas the inelastic nuclear scattering measures the phonons coupling to the iron at the active site of myoglobin, the inelastic Rayleigh scattering is determined by all phonons within the sample. In a second experiment the radiation was scattered by a hydrated met-myoglobin sample at T=62, 124, 189 and 300 K. Energy analysis was done in scattering direction by an ^{57}Fe-metal-foil. Radiation whose energy difference ΔE to the Mössbauer level is compensated by a phonon yields nuclear forward scattering in the iron foil with delayed intensity proportional to the phonon density.

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1-16

STUDY OF THE PROTEIN DYNAMICS BY OFF-RESONANCE ROESY EXPERIMENTS.

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Quantitative analysis of molecular internal dynamics is usually performed from NMR heteronuclear measurements. Nevertheless, it is possible to extract information on protein dynamics by homonuclear NMR spectroscopy, using the off-resonance ROESY. This experiment allows the observation of a relaxation phenomenon, which is the weighted sum of the NOESY and ROESY relaxations. In this presentation, we will describe the application of the off-resonance ROESY to the study of the internal dynamics of a protein and of a peptide. This experiment has permitted to measure the mobility of more than 150 vectors connecting hydrogens inside a calcium-binding protein of 108 AA, the parvalbumin. The obtained values are compared with results on parvalbumin dynamics obtained from ^{15}N and ^{13}C relaxation measurements. The protein backbone is found mainly rigid, with the exception of some residues the mobility of which can be inferred by independent experimental observations. The calcium loops and the domain located between them are mainly rigid. Some long-range vectors connecting AB protein domain and the other protein domains are found more mobile. These observations are analyzed in terms of the relative movements of protein domains and in terms of the mobility of residue sidechains.

The relaxation phenomenon observed in the off-resonance ROESY has been also analyzed in detail on a smaller molecule by recording experiments on endothelin, a vasoconstrictor peptide of 21 AA. This analysis has permitted to evaluate the relative influence of the spin diffusion and internal dynamics on the experimental observations.

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1-17

FUNCTIONAL DYNAMICS OF BACTERIORHODOPSIN STUDIED BY ELASTIC INCOHERENT NEUTRON SCATTERING AND HYDROGEN-DEUTERIUM LABELING. V. Réat, H. Patzelt, M. Ferrand, C. Pfister, D. Oesterhelt and G. Zaccai.

Elastic incoherent neutron scattering from a protein is strongly dominated by H nuclei. The variation of the elastically scattered intensity versus the scattering vector Q gives access to the mean square displacement $\langle u^2 \rangle$ of H nuclei, which, in the given time range (energy resolution) of the neutron spectrometer, reflects the movements of the groups to which H is linked. If H is replaced by D, the scattered intensity is lowered by a factor of 40, allowing in principle to observe selectively H-labeled parts of samples.

In previous experiments on fully H-labeled Bacteriorhodopsin (BR), the light-driven proton pump in *Halobacterium salinarium* , the analysis of $\langle u^2 \rangle$ with temperature revealed a "dynamical transition" from a harmonic behavior (at low temperatures) to non-harmonic movements of high amplitude at a temperature for which hydration water is melt (M. Ferrand, A.J. Dianoux, W. Petry, G. Zaccai, Proc. Natl. Acad. Sci. USA 90, 9668-9672, 1993).

First, we show an analysis of data obtained from scattering experiments in two different Q-ranges, revealing an heterogeneity in the mean global movements in BR

Secondly, we selectively H-labeled BR on retinal, Trp and Met, whereas all other aminoacids and lipids were D-containing. The H-region was chosen as to concern essentially the "retinal pocket" and the extracellular half of the transmembrane helices. We show that this region appears as much more rigid than the mean global protein : as compared with the fully H-containing sample, i) the observed movements remained of lower amplitude ; ii) the dynamical transition was upshifted by 80K. Moreover, this region appeared as shielded from solvent-melting effects.

The vectoriality of the proton transport in BR triggered by absoprtion of a photon by retinal could well accomodate this dynamical characteristics of a "hard core" in a "soft body".

This study shows also the feasability of elastic incoherent neutron scattering on biological samples with only a few percent of their natural content of hydrogens, allowing the description of the heterogeneity of movements inside a protein.

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1-18

TIME-RESOLVED FLUORESCENCE OF PROTEINS: UNUSUAL AGITATION EFFECT ON PROTEINS PHOTOPHYSICS. A.Ababou, and D. Gerard.(*)

Time-resolved fluorescence is often used to study the conformational dynamics of proteins. Single tryptophan containing proteins (STCP) have been found in many cases to show a heterogeniety in their fluorescence decays [1,2]. The photophysics origin of this complex fluorescence dynamics in STCP is still a subject of extensive and open debat in recent years. Indeed two principal explanations are invoked: **(i)** the proteins present multiple conformations substates which are interconverting at a rate slower than the decay rate [3,4,5], **(ii)** Trp residue, donor, undergoes a multiple deexcitation pathways due to an *enegry transfer* to a different acceptor sites in STCP [6].

Using the single photon counting technique with a Ti:Saphire laser and using iterative reconvolution method developped in our laboratory (IRM) [7], maximum entropy method (MEM) [8], and *energy transfer* method (ETM) [6], we have studied the fluorescence decay of two STCP models which have known 3D structures, RNaseT1 and Nuclease. In the absence of stirring solutions there was a contribution of a subnanosecond component. The interpretation of this result in this study suggests: **(i)** a photoproduct formation, **(ii)** a Trp residue regeneration by a recombination reaction with the solvated electron and **(iii)** the existence of a long range electron transfer. Finally, the difficulty to interpret this result demonstrate again the complexity of the excited state kinetics of Trp residue in STCP and thus the importance of more details that were needed to understand the heterogeniety origin in the fluorescence decays of proteins.

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1-19

STUDY OF THE PARVALBUMIN AFFINITY DIFFERENCE FOR CA^{++} AND MG^{++} USING FREE ENERGY DIFFERENCE CALCULATIONS.

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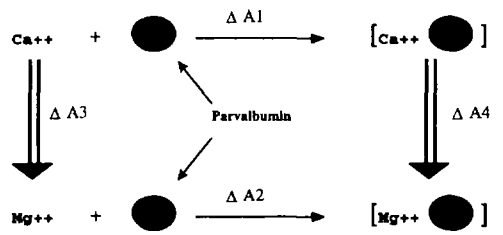
Recently, molecular dynamics simulations have been applied to the calculation of free energy differences, by taking advantage of the fact that a « non-physical » path can be followed step by step during a simulation . This work is an attempt to calculate parvalbumin - (which is a typical « EF Hand » protein) - binding energy difference between Ca^{++} and Mg^{++}.



One can see on the thermodynamic cycle above that it is possible to access to the free energy variation [ΔA2- ΔA1] (associated to the affinity difference of the protein for these cations) by the calculation of the free energy variation [ΔA4- ΔA3]. ΔA3 is obtained through the progressive transformation of the Ca^{++} in Mg^{++} in a solvation sphere. ΔA4 corresponds to the relative difference of free energy associated to the same cation transformation within the protein. We have followed the structural evolution of the surrounding of Calcium during its transformation in Magnesium, and this either in water (ΔA3) or in the protein (ΔA4). In both cases, the first solvation layer changes gradually from a structure with 8 oxygen neighbours for Ca^{++} to an octahedral organisation, with 6 neighbours, for Mg^{++}.

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1-20

COORDINATION OF PROSTHETIC GROUPS TO PROTEINS

E. Balog, R. Galántai, J. Fidy, *M. Köhler, *J. Friedrich

Proteins keep the machinery of life running, for instance as enzymes in catalytic reactions, as transport and storage proteins and in many other ways. Many of the proteins need prosthetic groups for proper functioning. It is an interesting problem to elucidate the relation between the state of the prosthetic group and the associated structure of the apoprotein.

In this work, this problem has been studied by spectral hole burning in Mg-mesoporphyrin substituted horseradish peroxidase (Mg-MP-HRP). Pressure tuning and Stark-effect experiments on spectral holes were carried out. In pressure tuning experiments the change of a spectral hole is measured under external pressure; the results give information about the vacuum absorption frequency of the chromophore and the isothermal compressibility of the protein. In Stark-effect experiments, the change of a spectral hole is measured under the influence of an external electric field; and the results carry information about the dipole moment of the chromophore, about its polarizability and about the local electric field generated by the surrounding protein.

The spectral bands in the Q-range unraveled the presence of two conformers of Mg-MP in HRP (and for both a Q_x-Q_y splitting could be detected). The pocket field breaks the effective inversion symmetry of the chromophore by inducing a dipole moment. Upon adding substrate to HRP, which binds in the heme pocket of the enzyme, only one conformer can be detected (with an increased Q_x-Q_y splitting). This substrate binding may cause a structural rearrangement around the heme that is felt as a changed pocket field leading to a different dipole moment.

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1-21

TWO ENANTIOMERS DISTINGUISH THE INACTIVE "CURVED" AND ACTIVE "STRAIGHT" TUBULIN STATES.

P. Barbier, V. Peyrot, D. Leynadier and J.M. Andreu.

Tubulin is a GTP binding protein which polymerizes into microtubules. This protein may exist in equilibrium of two conformational states : a "straight" active and a "curved" inactive form allosterically controlled by the nature of the nucleotide (1). GTP favors the active form whereas GDP favors the inactive form (2). We have investigated the interaction of two chiral isomers included in the colchicine ligand site family, in function of the occupancy in the tubulin nucleotide site. In glycerol free buffer, the R- and S-isomers induced the formation of abnormal structures from GTP-Mg-tubulin. When GDP-tubulin was used, only the S-isomer provoked an increase in optical density, indicating the formation of polymers. Electron microscopy of the polymers formed from GTP-Mg and GDP-tubulin indicated they had the same filamentous structures. HPLC analysis showed that an uncoupled GTP hydrolysis and an exchange of nucleotide were involved in the mechanism of abnormal polymerization induced by the R- and S-isomers. By analytical ultracentrifugation, we observed an inhibition of the Mg^{2+}-induced ring formation from GTP-Mg- and GDP-tubulin in presence of both isomers. The R- and S-isomer binding parameters were determined by fluorescence titrations. A 3-4-fold and a 1-2-fold decrease in the apparent affinity constant for GDP-tubulin relative to GTP-Mg-tubulin were found for the R- and S-isomers respectively. Analysis of these results by a system linking conformation and association equilibria allowed us to conclude that the S-isomer and in more important way the R-isomer are able to distinguish between the inactive "curved" and the active "straight" tubulin conformations.

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1-22

EFFECT OF MG-ION ON THE BINDING OF MESOPORPHYRIN TO HUMAN SERUM ALBUMIN AND LIPOSOMES. I. Bárdos-Nagy, R. Galántai, and J. Fidy

It is known that Human Serum Albumin (HSA) plays an important role in the transport, distribution and metabolism of porphyrin derivatives, forming stable complexes with them.

The elucidation of the mechanism of action of HSA in the binding processes is interesting from the point of view of diagnostic and therapeutic methods - Photodynamic Therapy (PDT) - based on the photophysical/photochemical properties and selective accumulation of porphyrin derivatives in tumorous cells.

In our study the binding of Mesoporphyrin IX (MP) and Mg-Mesoporphyrin IX (Mg-MP) were examined to the HSA, to DMPC/DMPG liposome, and to HSA-liposome system by fluorescence spectroscopic, and DSC methods. The free-base MP has represented the endogenous porphyrin, the Mg-MP has modeled the nowadays applied dyes for PDT, the bacteriochlorophyll and its derivatives, and the liposome has been applied to imitate the membrane structure.

The results suggest that the coordination of Mg ion into the porphyrin ring decreases the association constants compared to the free-base MP either in HSA-porphyrin either in liposome-porphyrin system.

The influence of the liposomes on the transport properties of HSA was also studied. On the bases of spectroscopic and DSC experiments, binding by hydrophobic interactions was found between HSA and liposomes. This binding effect showed different influence on the distribution of free-base, and Mg-porphyrin between the lipid and the protein.

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1-23

DOMAIN SPECIFICITY IN CALMODULIN-TARGET INTERACTIONS. A.Barth, S.R.Martin and P.M.Bayley.

The relative importance of the N- and C-terminal domains of calmodulin (CaM) in their interaction with skeletal muscle myosin light chain kinase (sk-MLCK) has been investigated using tryptic fragments of calmodulin (N-domain/C-domain) and synthetic target peptides derived from the CaM binding sequence of sk-MLCK ["WFF" (KRRWKKNFIAVSAANRFK) and variants including WF10 (WFF residues 1-10]. Near-UV circular dichroism spectra, graphically resolved into ^1L_a and ^1L_b components, show that the environment of the Trp residue is different in complexes with the N- and C-domains, and that a 1:1 mixture of the two domains binds to the full-length WFF in the same orientation as does intact CaM. Titrations of fluorescence and CD signals show that both domains can bind to both ends of the WFF peptide. The C-domain binds with ~ five-fold higher affinity to the N-terminal end of WFF (K_d ~ 14 nM) than to the C-terminal end (K_d = 40-80 nM). The N-domain binds to both ends of WFF with similar affinity (K_d ~ 150 nM). The short WF10 peptide binds to both CaM domains with some 100-1000-fold lower affinity than does WFF. The partial selectivity of the C-domain of calmodulin for the N-terminal portion of the target sequence, and the generally stronger interaction of the C-domain with the target sequence, suggest that this domain plays the key role in selecting the "correct" binding site on the sk-MLCK target sequence. Examination of the nmr and X-ray structures of the Ca_4-CaM-MLCK-peptide complex suggests that the second domain would bind in a two-fold symmetry-related position. This would further imply the existence of a similar relationship between key amino-acid residue side-chains in the two halves of the inherently polar target structure. Such a target sequence signature could be an indication for forming a compact complex structure with calmodulin.

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1-24

NMR CONFORMATIONAL STUDY OF THE CYTOPLASMIC DOMAIN OF THE CANINE SEC61γ PROTEIN FROM THE PROTEIN TRANSLOCATION PORE OF THE ENDOPLASMIC RETICULUM MEMBRANE. V. Beswick, F. Baleux, T. Huynh-Dinh, F. Képès, J.-M. Neumann and A. Sanson.

Conformational studies of the synthesized N-terminal cytoplasmic domain of the canine Sec61γ protein, an essential protein from the translocation pore of secretory proteins across the endoplasmic reticulum membrane, were performed using two dimensional proton NMR spectroscopy. This canine domain is one of the smallest domains within the homologous protein family and may thus constitute the minimal functional structure. The peptide was solubilized in pure aqueous solution or in the presence of dodecylphosphocholine micelles mimicking a membrane-solution interface. In pure aqueous solution, the peptide is remarkably unfolded. Forming a stable complex with dodecylphosphocholine micelles, it acquires a well defined α helix-loop-α helix secondary structure, with the first helix, highly amphipathic, lying at the micelle surface. The loop comprising four residues, is delimited by two flanking helix capping structures, highly conserved in the whole homologous protein family. No tertiary structure, that could have been revealed by inter-helix NOE contacts, was observed. From these experimental results and using general arguments based on sequence information and knowledge on peptide-membrane interactions, a structure of the entire Sec61γ protein in membrane bilayers is proposed.

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1-25

NMR STUDIES OF THE 3D STRUCTURE OF PROTEIN G4 FROM *DENDROASPIS POLYLEPIS POLYLEPIS*. J. Boisbouvier, J.P. Albrand, H. Schweitz, M. Lazdunski & D. Marion.

Protein G4, extracted from the venom of *black mamba* (*Dendroaspis p. p.*), contains 81 residues and five disulphide bridges. This protein does not belong to any class of toxin so far isolated from venom of *Dendroaspidae*, and its function has not yet soon determined.

Two dimensional ^1H homonuclear N.M.R. spectroscopy (TOCSY, NOESY), at three temperatures and two pH, was used for complete assignment of proton resonances. Assignment was confirmed by natural abundance 2D ^{13}C-^1H correlation experiments (HSQC and HSQC-TOCSY). To avoid the overestimation due to the linewidth, $^3J_{HNH\alpha}$ was extracted from COSY-DQF by iterative simulation of individual extracted 1D spectra. Comparison of the values of $^3J_{HNH\alpha}$ with the chemical shift of $H\alpha$, $C\alpha$ and $C\beta$ was used to fix backbone dihedral restraints. Two disulphide bonds were identified by proteolytic digestion and measurement of the amino acid mass of the fragments.

This information was used in combination with unambiguously assigned nOe for structure calculations using a simulated annealing protocol. The NMR structure of protein G4 is composed mainly of β-sheets and has no structural homology with snake toxins already studied. To obtain a higher resolution structure we are currently attempting to solve the disulphide bonding pattern using two approaches : biochemical investigation and structure calculations with the different disulphide bonding combinations.

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1-26

CRYSTAL STRUCTURE AND MOLECULAR DYNAMICS OF A *PHYTOPHTHORA CRYPTOGEA* PROTEIN ELICITOR-STEROL COMPLEX: G. Boissy, J.-Ph. Demaret, M. O'Donohue and S. Brunie.

Most species of the genus *Phytophthora* produce 10 kDa extracellular protein elicitors, generically named elicitins. These proteins induce leaf necrosis in infected plants and elicit an incompatible hypersensitive-like reaction, leading to resistance against fungal and bacterial plant pathogens. The crystal structure determination of β-cryptogein (CRY), secreted by *P. cryptogea*, was undertaken to identify structural features important for the necrotic activity of elicitins. The structural and dynamical knowledge of a member of the elicitin family may provide new approaches for agricultural pest control, making it possible to engineer a non-toxic elicitin capable only of eliciting plant defences.

The structure of CRY has been determined using the multiwavelength anomalous diffraction (MAD) technique and refined to 2.2 Å resolution (R=21.8%). The overall structure reveals a novel folding type made of six α-helices and a highly conserved beak-like motif composed of an antiparallel two-stranded β sheet and an Ω-loop. This motif is assumed to be a major recognition site for a putative receptor and/or ligand. Since the structure is not fully packed and exhibits several inward and outward cavities, molecular dynamics simulations in water were performed to study the induced local structural fluctuations. Furthermore, this structural feature allows the fitting of a ligand, and such a structure with a sterol molecule bound inside the protein's large hydrophobic cavity has been observed and refined to 2.15 Å resolution (R=19.6%). Molecular dynamics has also been used to check the possibilities to accommodate various ligands.

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1-27

THE HYDROLYSIS OF RIBONUCLEASE A: A QM/MM STUDY. A. Dejaegere, C. Burgess and M. Karplus.

The hydrolysis of phosphate esters in solution and enzymes is of great biological interest. One such reaction is the degradation of RNA by the enzyme Ribonuclease A (RNAse A). Although a wealth of structural and biochemical information exists for this enzyme, the mechanism of hydrolysis has not been fully elucidated.

As a first step in the investigation, we have employed a mixed quantum mechanical/ molecular mechanical (QM/MM) potential to the hydrolysis of a model system, dimethyl phosphate (DMP). *Ab initio* gas-phase calculations using high-level basis sets have been used to determine the reaction profile for the attack of OH^- on DMP. Semiempirical MNDO optimisation, using previously adapted parameters, of structures along the profile were performed.

The effect of solvent on the energetics of the reaction was studied by performing stochastic boundary molecular dynamics on solvated structures along the reaction profile. Polarisation effects between the phosphate and water could be determined by comparison with gas-phase results.

The verification of the QM/MM method suggests that it will be useful for future studies on the mechanism of RNAse A.

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1-28

INCRESED THERMAL STABILITY OF AN ENGINEERED ADENYLATE KINASE DESIGNED TO BIND A Zn^{2+} ION. S. Burlacu, V. Perrier, A.-M. Gilles, O. Bârzu, J. Mispelter and C. T. Craescu

Escherichia coli adenylate kinase (AKe) is a 214 residue protein having an α/β topology and an external domain (INSERT) containing a four-stranded β-sheet. We designed an artificial variant in which four residues in this last domain were substituted by cysteine residues (AK_{4C}). The new variant conserves the enzymatic activity and has the capacity to bind a Zn^{2+} ion. Large scale expression and purification of AK_{4C} enabled us to analyze its structural and physicochemical properties. 1D and 2D NMR comparative studies of the wild-type and engineered proteins demonstrated that the global structure and the β-sheet topology is not significantly perturbed by the four substitutions. The Zn-binding unit has a specific structure which is different of all the known Zn-finger domains. NMR, enzymatic and calorimetric experiments showed that AK_{4C} exhibits a substantial increase in thermal stability: the mean denaturation temperature (T_m) increases by about 11°K relative to the wild-type protein. Amide hydrogen exchange monitored by 2D NMR showed that the core β-sheet exchanges significantly slower than the INSERT β-sheet in both wild-type and engineered molecules. In order to test the structural stability and autonomy of the external domain we synthesized a 45 residues peptide covering the INSERT sequence. CD and NMR studies revealed that the peptide has a largely flexible, poor-structured conformation. Adding 30% (v/v) trifluoro-ethanol in the solvent increases the secondary structure content of the peptide. The present results indicate that the INSERT structure in the intact AKe molecule is critically stabilized by its anchoring to the central (core) domain. We can therefore argue, that in a similar manner, Zn-binding in the engineered variant brings an additional stabilization, contributing to the increase of the thermal stability of the whole protein.

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1-29

STRUCTURAL ANALYSIS OF KERATIN INTERMEDIATE FILAMENTS. B. Busson, J. Doucet.

Keratin fibres have been studied for years but their structure at the microfibrillar level, as for all the other intermediate filaments, is still not well known. More precisely, little is understood about the way keratin molecules are packed into a microfibril.

From high resolution X-ray diffraction data collected on synchrotron sources (LURE and ESRF), we have tried to improve the model of keratin intermediate filament organization. Data sets from several samples and under various conditions (humidity and temperature) have been used. The analysis has been performed by computer modelling and numerical simulation, taking into account biochemical data. Simulated X-ray diffraction 2D-pattern give information ranging from the alpha-helix conformation inside the coiled coil ropes to the microfibrillar packing.

We have developped new models concerning:
- the conformation of protein side chains into the coiled coils,
- the packing of coiled coil molecules into a microfibril,
- the lateral organization between microfibrils.

We have shown that computer modelling, along with numerical simulations, is a powerful tool to analyse qualitatively and quantitatively diffraction patterns from keratin fibres. The methods used can be extended to many other fibrous materials.

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1-30

STRUCTURAL PROPERTIES OF AN UNFOLDED PROTEIN.

V. Receveur, D. Durand and P. Calmettes.

Phosphoglycerate kinase (PGK) is completely unfolded by guanidinium chloride (GdmCl) at molarities higher than 1 M. In these conditions tryptophan-fluorescence and circular-dichroism (CD) measurements suggest that the protein is very likely devoid of secondary structure. Neutron scattering profiles provide additional important information about the global structure of denatured PGK. In particular they show that the protein is very swollen and behaves as a random coil with excluded volume interactions. This means that the conformational space available to the protein is already partialy restricted in the unfolded state. The contour length of the chain is found to be (138 ± 6) nm. This value corresponds to an almost extended β conformation whose contour length would be 143 nm. Consequently the totally unfolded protein is actually devoid of any local structure. Furthermore the statistical length, which reflects the stiffness of the molecule, is found to be (2.2 ± 0.2) nm. The statistical length is the shortest length of a chain segment allowing one end of the segment to be freely oriented with respect to the other one. The experimental value is in very good agreement with the fact that slightly more than 6 residues are required for orientation correlations to vanish. This is the first measurement of the rigidity of a polypeptide chain.
At lower GdmCl concentrations, that is between 1.0 M and 0.8 M, the protein remains unfolded. However the scattering spectra show that the polypeptide chain becomes helical with a very large pitch compared to the one of an α helix. According to CD measurements that helix is right-handed. This twisted conformation is probably the first step of the protein folding pathway.

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1-31

AP-LYASIC ACTIVITY OF THE FPG PROTEIN: AP-SITE ANALOGUES AND STRUCTURAL IMPLICATIONS. B. Castaing, J.L. Fourrey, N. Hervouet, S. Boiteux & C. Zelwer.

The FPG protein is a procaryotic DNA repair enzyme specific for damaged purines (FAPY residues and 8-oxoguanine). The FAPY residues result from ionizing radiations whereas 8-oxoguanine may result from oxydation by reactive oxygen species. Their effects are either genotoxic or mutagenic. Especially, 8-oxoguanine induces G-T transversions at a frequency of 50%. Excision of the damaged base leaves an abasic site which is also processed by the enzyme through successive β– and δ–elimination reactions which cleave DNA at at 3' and 5' sides of the lesion. The transition complex is a covalent imino intermediate between the abasic site and an amino group of the enzyme. Abasic site analogues are high affinity ligands of the protein. We compared the affinities of the *E. coli* and *L. lactis* enzymes for various abasic site analogues and demonstrated that the open-ring form of the ribose (reduced abasic site) is two order of magnitudes better a ligand than the heterocyclic form (tetrahydrofurane). Therefore, the transition complex results first from the excision of the base and hydrolysis of the heterocycle and second, from the imino intermediate formation. Chemical reduction of the covalent complexes leading to dead-end products can be done at β– and δ–elimination steps. The latter step is better explained if the reactive amine is the N-terminal proline of the protein which allows an enamine intermediate in equilibrium with an unstable Schiff base.

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1-32

INVESTIGATION OF TET REPRESSOR TRP-43 FLUORESCENCE UPON ANHYDROTETRACYCLINE BINDING: A MOLECULAR MECHANICS STUDY. M. Chabbert, P. Silvi Antonini, P. Alberti, W. Hillen, N. Ettner, W. Hinrichs, P. Fantucci, S. Doglia and H. Lami.

The Trp residue is widely used as a fluorescent probe of protein structure and dynamics although interpretation of data in terms of conformation is usually difficult. We present here a molecular mechanics analysis of Tet repressor Trp-43 fluorescence, which enlightens the usefulness of molecular mechanics methods to correlate Trp fluorescence with structural parameters. Trp-43 is located in the recognition helix of TetR operator binding domain. The maximum entropy analysis of the fluorescence decay of Trp-43 in F75 TetR and in its complex with anhydrotetracycline (AnTc) indicates that decay data can be described by the sum of three exponential components whose amplitudes are markedly altered upon AnTc binding. The adiabatic mapping of Trp-43 χ_1-χ_2 isomerization shows the existence of three main rotamers for the Trp-43 side chain. Examination of the rotamer environments indicates that the backbone carbonyl groups are the main potential quenchers and allows lifetime assignment. One of the rotamers may interact with His-44. The change in the rotamer distribution upon binding might be related to a conformational change of TetR N-terminal tail altering this interaction.

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1-33

X-RAY STRUCTURAL STUDY TO 2.2Å RESOLUTION OF HERPES SIMPLEX THYMIDINE KINASE IN COMPLEX WITH DEOXYTHYMIDINE AND A RANGE OF NUCLEOSIDE ANALOGUES. M.R.Sanderson, M.Bennett, L.N.Champness, F.Wien, R.Visse, E.DeClerq*, P.Herdewijn*, T.Ostrowski* & W.C.Summers**.

We have now refined to 2.2Å resolution the X-ray crystal structure, originally reported by D.G.Brown et al. (Nature Structural Biology, **2**, 876-880, 1995), of the thymidine kinase (TK) from herpes simplex virus type-1, both in complex with deoxythymidine (dT) and with ganciclovir. In order to better understand the factors which affect binding to this drug-target enzyme, we have also carried out X-ray studies, under cryocooling conditions, of the TK/aciclovir complex, and have solved and refined the X-ray structures of the enzyme in complex with several nucleoside analogues incorporating halogen substituents, ligands which have a range of inhibitory potencies against the enzyme. With water structure now mostly determined, it is possible to make detailed comparisons of the binding of these ligands. The interactions of the various deoxyribose or sugar-analogue moieties are a relatively constant feature of TK-ligand binding. By contrast, the purine or pyrimidine-analogue moieties exploit complementary regions of the active site, a site large enough to accommodate quite distinct modes of binding. The presence of a bulky substituent group in some of the analogues does not cause serious perturbation to the binding mode, except for a slight movement of one active-site side-chain.

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1-34

FLUORESCENCE SPECTROSCOPIC STUDIES ON THE MECHANISM OF THE MONOMER/DIMER TRANSITION OF ENZYME I OF THE PHOSPHOTRANSFERASE SYSTEM. F. Chauvin, D. Toptygin, S. Roseman and L. Brand.

In *E. coli*, glucose (preferred sugar) is transported *via* the phosphotransferase system (PTS). This enzymatic complex consists of four proteins. The PTS has an additional role: it regulates the expression of certain non-PTS genes, enzymes and transporters. Very importantly, the PTS itself is stringently regulated. How? Perhaps through Enzyme I (EI), the first of the four proteins. EI undergoes a monomer / dimer (M/D) transition (M= inactive / D = active). *In vivo* the M/D content varies greatly depending on the ligands present. The kinetics of interconversion is very slow: it takes 15 min. for completion at room temperature. Why? Are there changes in tertiary structure associated with M/D ?

It has not been possible to crystallize the whole protein and EI is too large for NMR studies. Therefore we made use of the techniques of fluorescence spectroscopy and of the intrinsic tryptophan (Trp) fluorescence of EI.

Wild type EI (WT) contains two Trps, at positions 357 and 498 respectively. In order to study changes at a single site in EI, we made three site-directed mutants: W357F, W498F and the double mutant. They dimerize and are active in the PTS assay (sugar phosphorylation).

The environment of the Trps was studied by time-resolved fluorescence intensity decay. Both single-Trp mutants 'sense' the M/D transition. This allows K_{eq} determination: K_{eq} is ~ 5 x 10^6 M^{-1} for the two single-Trp mutants.

Energy transfer allows the determination of the distance between two selected sites. The donor is Trp (at 357 or 498, depending on the chosen mutant). The acceptor is pyrene, specifically attached at Cys-575. For W498F the efficiency of energy transfer is different in M and D.

These two sets of data could be explained by a conformational change associated with the M/D transition.

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1-35

AFM- and TDPAC-Studies of Stellacyanin on 1T-TaS_2

B. Ctortecka, V. Hartmann, W. Tröger, M. Lösche and T. Butz.

Stellacyanin (Sc) is a small blue copper protein (MW 20 kD) that contains one single copper ion bound in the unique "type 1" site which is also found in other proteins involved in electron transport (e.g. azurin, plastocyanin). In earlier work, using time differential perturbed angular correlation (TDPAC) of γ-rays, we determined the strength and symmetry of the electric field gradient tensors (EFG) at type 1 sites of several blue copper proteins in which Cu was isomorphously replaced by the radioisotopes ^{111m}Cd and ^{199m}Hg. According to these studies the type 1 coordination is dominated by a trigonal-planar ligation of the metal by a cysteine and two histidines. In some proteins one or two further axial ligands (methionine and/or glycine) appear, but exert only little influence on the metal. A deeper understanding of the coordination requires the full characterisation of the EFG, i.e. in addition to strength and symmetry its orientation and sign has to be determined. To prepare oriented protein monolayers we incubated atomically flat surfaces of the layered transition metal dichalcogenide 1T-TaS_2 with KPP buffered Sc (rhus vernicifera) and found that the protein adsorbs readily at pH ≈ 8. Atomic force microscopy (AFM) showed the deposition of protein clusters on the surface (patches with diameters of ~1-3 mm with internal structure on a ~30-50 nm length scale). TDPAC of ^{111m}Cd-derivatives of Sc on 1T-TaS_2 revealed that the protein is adsorbed without preferential orientation. Therefore, to achieve our aim of oriented adsorption we currently use two alternative strategies: (i) Mutants with a surface cysteine residue are expected to form well defined bonds on atomically flat Au surfaces. (ii) Biotinylated Sc is expected to bind to polyelectrolyte interfaces on substrates functionalized with streptavidin.

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1-36

X-RAY CRYSTALLOGRAPHY STUDIES IN SOME TRANSTHYRETIN VARIANTS - THEIR IMPLICATIONS IN AMYLOIDOGENESIS. A. M. Damas, M. P. Sebastião, M. J. Saraiva, Z. Dauter and V. Lamzin.

Amyloidosis is a generic term for a series of diseases related to the presence of protein aggregates in the extracellular tissues and, depending on the protein, different amyloid pathologies have been described. In the case of Familial Amyloidotic Polineuropathy (FAP), Portuguese type, a variant transthyretin is the major protein component of the amyloid deposits. Other amyloidogenic TTR variants have been found and in most cases they present only one point mutation. We think that the replacement of one aminoacid for the other induces a structural change that promotes the aggregation of the protein.

While the electron microscopy photographs on FAP fibrils indicate that the units composing the fibrils are smaller than the TTR tetramer, the X-ray crystallography studies on three variant TTRs point to a possible destabilization of the tetrameric protein, which is consistent with the presence of a monomer or dimer in the fibrils. We believe that detailed structural analyses of the variants will contribute to establish the conditions that will impair protein aggregation.

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1-37

DETERMINATION OF THE EXCITED-STATE LIFETIMES OF TRYPTOPHAN RESIDUES VIA IMPROVED MULTIFREQUENCY PHASE FLUOROMETRY: APPLICATION TO BARNASE. K. De Beuckeleer, A. Sillen and Y. Engelborghs.

To measure more accurately fluorescence lifetimes the multifrequency phase fluorometer as described by Clays et al (1989) was upgraded with a BeamLok 2080 Ar^+ ion pump Laser (Spectra Physics) that pumps a mode-locked titanium-doped sapphire laser (Spectra Physics Tsunami). The Tsunami pulses have a pulse width of less than 2 ps and a repetition frequency of 80 MHz. To lower the repetition frequency to 800 kHz a pulse selector was used. The wavelength of 295 nm was reached by optical frequency tripling. The detection system of the previous instrument was replaced as described by Rita Vos et al. (1995)

Barnase is a small, monomeric, single domain enzyme. It contains three tryptophan residues at positions 35, 71 and 94, respectively. Excited-state lifetimes of the tryptophan residues of wild-type barnase and tryptophan mutants were determined using the new apparatus and compared to previous data (Willaert et al, 1992). Measurements were performed at two pH values (5.8 and 8.9), close to the extremes of the fluorescence titration of barnase. The lifetimes found are essentially the same as previous data although we found for each mutant small contributions of additional lifetime components. The major lifetime of tryptophan 71 was determined by making the single tryptophan mutant and found to be correctly predicted.

References:
Clays K., Jannes J., Engelborghs Y. and Persoons A., *J. Phys. E: Sci. Instrum.* 22 (1989), 297-305.
Vos R. and Engelborghs Y., *Biochemistry* 34 (1995), 1734-1743.
Willaert K., Loewenthal R., Sancho J., Froeyen M., Fersht A. and Engelborghs Y., *Biochemistry* 31 (1992), 711-716.

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1-38

NESTED INTERACTION CAN DESCRIBE THE ATP-BINDING BY GroEL. N. Hellmann and H. Decker

The interaction of the chaperonin GroEL with GroES and ATP facilitates protein folding in E. coli and is accompanied by a cooperative ATPase activity. The degree of cooperativity depends on the concentration of K-ions and GroES. An appropriate model for the conformational transitions is therefore crucial to understand the mechanism responsible for the chaperonin's activity. Recently the ATPase activity of 2x7-meric GroEL was described by a nested KNF allosteric model (Yifrach & Horowitz, 1995). We applied a different nesting model in which not only the interactions within but also between the allosteric units are described in a MWC-like manner. No negative cooperativity is included in our model. According to our model four conformations for the heptamer are possible. These conformations are characterized by their binding constants and rates of hydrolysis. The transition between the conformations are given by the appropiate allosteric equilibrium constants, which reflect the hierarchical composition of the molecule. So we could distinguish between effector mediated (allosteric equilibrium constants) and protein characteristic equilibrium constants (ATP binding and rate of hydrolysis). This model is successfully tested by describing several data sets from the literature appropriately.

Yifrach O, Horowitz A (1995) Nested cooperativity in the ATPase activity of the oligomeric chaperonin GroEL. Biochemistry 34:5303-5308

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1-39

PROTEIN STRUCTURE PREDICTION AND MOLECULAR BIOCOMPUTING. APPLICATIONS TO PROTEIN "DOMAIN DESIGN".

G. Deléage, C. Blanchet, C. Combet, F. Penin & C. Geourjon

We have developed a new method to predict the secondary structure of proteins from their sequences. The method called SOPMA for Self-Optimised Prediction from Alignment uses (i) the information contained into the multiple alignment of related protein sequences, (ii) the auto-optimisation of predictive parameters on a reference database. The method has a global performance of about 70% of correctly predicted residues when checked onto 130 proteins with known 3D structures and less than 25% sequence identity. When SOPMA is used in combination with others methods based on neural netwoks, the success is 82% for 75% of jointly predicted residues. In order to offer to the biologists integrated tools for protein sequence analysis, programs such as ANTHEPROT or PSA have been developed. These programs run either on PC or workstations (SGI, IBM, SUN) computers and are available by anonymous ftp to ftp.ibcp.fr. Moreover a WWW interface to secondary structure prediction methods, fasta, sites and signature detection algorithms as well as multiple alignment programs has been developed. (http://www.ibcp.fr/predict.html). Up to now more than 20000 requests have been analyzed on our server which is supported by academic funds. All these tools are used in our lab to perform a **"domain design approach"** in 3D structure determination by NMR. Two examples of such a strategy will be given on two DNA binding domains. Preliminary results concerning the prediction of 3D protein structure from sequence by a combination of distance geometry database sampling and simulated annealing will be presented.

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1-40

PHYSICO-CHEMICAL CHARACTERIZATION OF AN EXTRACELLULAR HEME-BINDING PROTEIN, HASA. N. Izadi, C. Wandersman, M. Delepierre, A. Lecroisey.

We have been studying the conformational properties in solution of carboxy-terminal signal sequences involved in the protein secretion in gram negative bacteria to understand transport mechanism at the molecular level. One such protein HasA, a proteineous siderophore secreted by the bacteria *Serratia marcescens,* is able to bind free heme as well as to capture it from hemoproteins. HasA is secreted by under iron deficiency conditions. In fact, many bacterial hemoproteins involved in heme acquisition have been isolated recently, comprising outer membrane receptors and extracellular heme-binding protein. The mechanisms by which these proteins extract heme have not been described up to now. HasA does not present sequence similarities with other known hemoproteins suggesting that it possesses a new type of heme binding site. The main physicochemical properties of HasA, whose knowledge is essential for understanding its function, have been characterized. HasA is a monomer of 19 kDa, that binds one *b* heme per molecule with a high affinity. The EPR spectra indicate that the heme iron is in a low-spin ferric state, and that the two iron axial ligands are His and His^-. The low oxidation-reduction potential value (-550 mv vs. SHE) of the heme bound to HasA suggests that heme could be exposed to the solvent. According to circular dichroism data, the binding of heme does not seem to modify the conformation of HasA. The conformation of the C-terminal region has been probed by proton NMR.

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1-41

FLUORESCENCE LIFETIME DISTRIBUTIONS IN Na, K-ATPase: MOLECULAR RELAXATIONS AND INTER-TRYPTOPHAN ENERGY TRANSFER. A. P. DEMCHENKO[1], J. GALLAY[2], M. VINCENT[2] and H.-J. APELL[3].

The analysis of fluorescence lifetime distributions for Na,K-ATPase, a large membrane protein containing 16 Trp residues, allows to observe new important features. No components in emission decay kinetics are detected with lifetimes shorter than 0.3 ns. In nanosecond time range, instead of expected broad lifetime distribution due to averaging of contributions from individual Trp residues and their conformational sub-states, we observe discrete narrow lifetime maxima, the positions and relative intensities of which depend strongly upon the emission wavelength. The shortest component 0.3-0.4 ns exhibits sign reversal at the long-wavelength edge of emission spectrum, which is a signature of dielectric relaxations in this time range. The second component with lifetime about 1 ns may be also of relaxational origin, since it is strongly sensitive to the change of temperature and vanishes on increase of emission wavelength. In contrast, the longest component of emission decay in the range of 5-7 ns increases substantially in relative magnitude and lifetime value on transition from short-wavelength to long-wavelength emission. This component may be attributed to fluorescence of tryptophans which are acceptors in excitation energy transfer.

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1-42

ORIGIN OF THE PHOTO-ACOUSTIC SIGNAL IN CYTOCHROME P-450$_{cam}$: ROLE OF THE ARG186-ASP251-LYS178 BIFURCATED SALT-BRIDGE. C. Di Primo, E. Deprez, S. G. Sligar & G. Hui Bon Hoa.

The origin of the photo-acoustic signal in ferrous CO-camphor-cytochrome P-450$_{cam}$ was investigated. We considered the hypothesis that electrostriction resulting from the transient exposure of the Arg186-Asp251-Lys178 bifurcated salt-bridge, which would control the diffusion step of the substrate, could be responsible for part of the photo-acoustic signal. First we examined the effects of a site-directed mutation: the Asp251 residue was replaced by an asparagine, and then we changed the ionic strength of the medium. Upon the mutation the overall enthalpy and volume change of the CO dissociation reaction decrease from -5 kcal/mol to -24 kcal/mol and from 11 ml/mol to 5.4 ml/mol, respectively. Increasing ionic strength has the same effect on the thermodynamic parameters as the mutation, only for the wild type protein. This suggests that electrostriction around the bifurcated salt-bridge contributes to the acoustic signal. Following photo-dissociation of CO and diffusion of the molecule through the protein matrix, the structure would relaxe and the bifurcated salt-bridge would desolvate.

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1-43

CALCULATION WITH EXPLICIT SOLVENT SIMULATION OF PATHWAYS FOR THE CONFORMATIONAL TRANSITION BETWEEN THE GTP AND GDP BOUND STATES OF HA-*RAS*-P21. J.F. Díaz, Wroblowski B., Schlitter, J. and Engelborghs, Y.

The transitions between the water equilibrated structures of the GTP and GDP forms of Ha-*ras*-p21 have been calculated using the targeted Molecular Dynamics (TMD) method (Schlitter et al. 1993, *Mol. Sim.* 10, 291-309) both *in vacuo* and with explicit solvent simulation. These constrained molecular dynamics calculations result in different pathways depending on the nucleotide bound. Each pathway consists in a sequence of transitions affecting six segments of the protein, four of them forming a hydrophilic cleft around the nucleotide. The transitions are initiated by the removal or introduction of the γ-phosphate of the nucleotide, and proceed sequentially, crossing several low energy transition states. The movements are transmitted either by direct interactions between the segments or through the nucleotide. The GTP to GDP pathway is initiated by the removal of the nucleotide γ-phosphate. This gives some space to Gly12, Gly13 and Val14. Their movement is transmitted to the target recognition domain and the switch II region, forcing these segments to adopt another position. In a second step the target recognition domain and the switch II region undergo conformational transitions to reach an intermediate conformation. Finally there is a relaxation of the target recognition domain to its final state, that forces the switch II region to reach its target conformation. The calculated pathways allow the identification of many residues that play an important role in the conformational changes, explain the altered transformation properties of some and suggest mutations to alter the pathway.

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1-44

DYNAMICS OF c-*erb*B-2 TRANSMEMBRANE DOMAIN. J.-P. Duneau, D. Genest and M. Genest

The *neu/erbB-2/HER2* gene encodes a transmembrane receptor tyrosine kinase of the family of the EGF-R. Genetic evidence shows that a point mutation in the predicted transmembrane domain is involved in the constitutive activation of the enzyme potency. To explore structure/dynamics-activity relationships for this membrane protein, molecular dynamics simulations have been applied to the whole transmembrane domain. Starting from α helix conformations, a flexible behaviour corresponding to a cooperative transition that locally changes the α helix pattern towards a π helix one was observed in vacuum simulations.
In the present work, we demonstrate that the transition phenomenon does not depend upon experimental conditions as force field or side chain orientations. Particularly, the role of the helix-destabilizer residues (Val, Ile) is emphasised by performing additional simulations on a modified sequence with Val/Ala substitutions. We also investigate the influence of membrane environment on the dynamics of the wild c-*erb*B-2 transmembrane peptide. A model was built in which the α-helical peptide is surrounded by a 52 di-lauryl phosphatidyl ethanolamine bilayer and hydrated with 1036 water molecules. Two 500 ps molecular dynamics simulations have been produced after 100 ps equilibrium periods. One shows the appearance and the propagation of a π deformation similar to those detected in vacuum simulations. The α→π helix transition is not an artefact of the vacuum environment. It is rather the consequence of the c-*erb*B-2 transmembrane sequence. Vacuum approximation seems suitable to mimic the core of a membrane although differences in motions are evidenced. The structural effects caused by the transition are sufficiently huge to modulate transmembrane signalling by c-*erb*B-2 known to be mediated by a dimerization process. This study also suggests that modelling of transmembrane domains as rigid α helices is questionable.

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1-45

CONFORMATIONAL STUDIES OF SYNTHETIC SUBSTRATES OF THERMOLYSIN BASED ON MOLECULAR MODELING AND ^{1}H-NMR. F.-Y. Dupradeau, A. Aboubeker, S. Baltora-Rosset, E. Pauthe, V. Larreta-Garde, G.A. Gacel, J.-P. Monti.

Thermolysin is a thermostable calcium-binding zinc endopeptidase commonly used as model of metalloprotease family. Thus, studies of thermolysin-substrate recognition yield molecular informations about the catalysis mechanism of peptide bond hydrolysis. In a previous work, use of synthesic substrates in a modified micro-environment (H_2O and glycerol) have led to an improved approach to thermolysin-peptide recognition. Thus, without minimizing the role of micro-environment on conformational changes and biological activity of enzyme, the conformations of synthetic substrates with various backbone lengths are investiged in H_2O/D_2O (9/1) and glycerol-d5 (5M) using 2D-NMR and molecular modeling. A lack of convincing solvent effect is evidenced on backbone atoms and on side-chains in spite of the high viscosity of glycerol. However, the comparison of structure families, obtained both by molecular modeling and ^{1}H-NMR data, with the structures of bound ligands in the active site, might explain most of the cleavages observed in the synthetic substrates from their structural parameters.

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1-46

SALT EFFECTS ON THE STABILITY OF MALATE DEHYDROGENASE. C. Ebel, P. Faou, B. Kernel, D. Madern, G. Zaccai

The stability, solubility and interactions of proteins are known to be affected by salts. The mechanisms of these effects are not well understood. They are related to an increase of surface tension, producing preferential hydration, and/or to specific interactions, even if weak, between water, ions, and protein groups. Halophilic malate dehydrogenase (hMDH)is is not only active and stable at high salt concentrations but unfolds at lower salt concentrations (2M KCl). Its three dimensional structure (Dym *et al.*, 1995, Science 267:1344) shows a large excess of 156 acidic residues on basic ones, mainly localised at the protein surface. In NaCl and KCl solutions, hMDH is solvated not only by water, but also by salt (Bonneté *et al.*, 1993, J. Chem. Faraday Trans. 89:2659). Studies of its stability has suggested different stabilisation mechanisms of the folded structure depending on the solvent (Bonneté et al., 1994, J. Mol. Biol. 244:436). Mutation of surfacic acidic residues affects the protein stability (Madern et al., 1995, Eur. J. Biochem. 230:1088). The aim of this study is to differentiate the respective effects of anions and cations on the stability and solvent interactions of hMDH. Fluorescence and residual activity show that with no salt, hMDH unfolds. When increasing the salt concentration -in the decimolar to the molar range-, folded hMDH is stabilised. The effectiveness of anions and cations for stabilising the active folded state is F,SO4>OAc>Cl; Ca>Mg>NH4,Na,Li,>K>Cs. The effectiveness of anions and cations for destabilising the active folded state at higher salt is I>Br>Cl>F,OAc,SO4; Ca>Mg>NH4,Na,Li,>K>Cs. The presence of very stabilising anions or cations masks the effects of the nature of the counter-ion. These effects can be interpreted in terms of ion binding on the protein surface and/or protein interior. Preferential interactions of folded hMDH with the salt, measured by neutron scattering are found to be significantly different depending on the nature of the salt.

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1-47

STRUCTURAL STABILIZATION OF BOTULINUM NEUROTOXINS BY TYROSINE PHOSPHORYLATION. J. A. Encinar, A.V. Ferrer-Montiel, A. Fernández-Carvajal, B.R. DasGupta, J.A. Ferragut, M. Montal and J.M. González-Ros.

Botulinum neurotoxins (BoNTs) are a family of bacterial proteins that produce flaccid paralysis in skeletal muscles by blocking Ca^{2+}-evoked exocytosis. BoNTs are proteins of 150 kD, composed of a heavy chain of 100 kD and a light chain of 50 kDa. The light chain is a metalloprotease that cleaves proteins involved in neurosecretion. The *in vitro* lability of pure BoNTs contrast with their exceptional stability inside the cells, suggesting their potential intracellular modification. Indeed, we recently discovered that tyrosine phoshorylation of BoNTs drastically increases both their catalytic activity and thermal stability (Ferrer-Montiel et al. (1996) J. Biol. Chem. *271(31)*:18322-18325). Here, we use FTIR spectroscopy and report that tyrosine phosphorylation of two different serotypes of BoTNs (BoNT A and BoNT E) increases by ~20% the content of α-helix secondary structure as evidenced by analysis of the conformationally-sensitive Amide I band. This structural change is accompanied by an increase in the Amide II band remaining upon exchange with D_2O, suggesting a much tighter packing of the phosphorylated protein, which prevent the H-D exchange and may justify its higher stability against thermal denaturation. Therefore, the functional modulation of BoNTs by tyrosine phosphorylation correlates with a profound structural stabilization. BoNTs are a family of bacterial proteins in which both, their structure and function have been found to be critically modulated by tyrosine phosphorylation.

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1-48

THE STRUCTURAL RELAXATION OF MYOGLOBIN AFTER REDUCTION AT LOW TEMPERATURE. D.C. Lamb, A. Ostermann, N. Engler, V.E. Prusakov and F. Parak.

The formation of intermediate, metastable states is necessary for many proteins to perform their function. To investigate the structural relaxation of these intermediate states, we have reduced met myoglobin at low temperature photochemically (20 K) or with x-rays (90 K). Upon reduction, an intermediate state is formed where the water molecule is bound to the heme iron in a 2+ low spin configuration, but the structure of the protein is frozen in the met conformation. To verify the structure of the intermediate state, x-ray diffraction and Mössbauer spectroscopy have been performed. The structure of the intermediate state is similar to that of met myoglobin and the water molecule is still bound to the 2+ low spin heme iron.

Above 150 K, a relaxation within the intermediate state is observable as well as the structural relaxation to the deoxy conformation. These protein relaxations have been monitored optically as a function of time and temperature from seconds to hours and from 160 K to 190 K. The structural relaxation is non-exponential in time and cannot be described by a static distribution of barrier heights.

The relaxation of the intermediate state to deoxy Mb has been measured in both sperm whale and horse myoglobin. The relaxation in horse myoglobin is faster than in sperm whale myoglobin.

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1-49

LIGHT SCATTERING INVESTIGATION OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE SUPRAMOLECULAR STRUCTURE

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Earlier it has been suggested that the activation of smooth muscle myosin light chain kinase (MLCK) can be modulated by formation of supramolecular structures [Sobieszek, A. (1991), J. Mol. Biol. 220:947-957]. Data obtained in this study demonstrate, virtually for the first time and by direct methods of light scattering, that inactive (calmodulin-free) MLCK apoenzyme exists in solution as a mixture of oligomeric, dimeric and monomeric species, which relative concentrations constitute at physiological ionic strength correspondingly 2, 53 and 45 weight percent. These long-living assemblies, lifetime of which is measured by minutes, are maintained in equilibrium. After activation of the kinase by calmodulin (CaM) we could not detect any appreciable changes in the distribution of the kinase species when either the kinase or CaM were present in molar excess. Analysis of the data obtained indicates that the kinase oligomer structure may be interpeted as a kind of flexible spiral in which MLCK molecules associate in head-to-tail fashion with the limiting configurations being a ring, and a long rod. It is important to point out that spiral-like oligomer fits very well to the helical structure of self-assembled myosin filaments [Sobieszek, A. (1972), J. Mol. Biol. 70:741-744].

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1-50

FLUOROMETRIC STUDY OF CONFORMATIONAL CHANGES OF THE MITOCHONDRIAL ADP/ATP CARRIER. C. Fiore, C. Schwimmer, A. Le Saux, P. Roux, C. Gourdet, F. Noël, V. Trézéguet, A.-C. Dianoux, P.V. Vignais, G.J.-M. Lauquin and G. Brandolin.

The adenine nucleotide carrier (Ancp) is a nuclear-encoded protein of the mitochondrial inner membrane, that catalyses the exchange of ADP and ATP between the matrix and cytosolic compartments. A strain of the yeast *Saccharomyces cerevisiae* expressing the sole Anc2p isoform of the carrier has been constructed and characterized. Conformational changes of isolated Anc2p have been studied by a fluorometric approach. Intrinsic fluorescence of Anc2p increases upon addition of ADP or ATP, all other nucleotides being inefficient. This effect can be inhibited or reversed by carboxyatractyloside (CATR), a specific inhibitor of transport, added before or after ADP or ATP. Bongkrekic acid (BA), another specific inhibitor of transport, enhances the ADP(ATP)-induced fluorescence signal. These fluorescence changes reflect the transition of the carrier between different conformational states, which probably occurs during ADP/ATP transport process. To further characterize these changes, the three tryptophanyl residues of Anc2p have been sequentially replaced by tyrosine. The resulting variants display biochemical and functional properties similar to those of Anc2p. Each individual tryptophanyl residue exhibits fluorescence changes in response to ADP(ATP), CATR and BA. However, differences in the specific fluorescence responses of the variants indicate that the tryptophanyl residues located in the N-terminal moiety of Anc2p are more sensitive to ADP(ATP) and BA binding to the carrier, while those of the C-terminal moiety sense preferably CATR binding. A mechanistic model is proposed based on the transition of Anc2p between different conformational states.

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1-51

STRUCTURAL ANALYSIS OF RECOMBINANT ANTIHAEMOPHILIA A FACTOR (FVIII) BY VARIOUS SPECTROSCOPIC METHODS. [1]N. Bihoreau, [2] M.P. Fontaine-Aupart, [3]J.M. Schmitter, [1] F. Dhainaut, [1] P. Paolantonacci.

Factor VIII is a plasma glycoprotein whose deficiency or absence causes a bleeding disorder, haemophilia A. FVIII-ΔII is a deleted variant of recombinant human FVIII, virally safe. This protein, active *in vitro* and *in vivo* in hemophilic dogs, is developed for haemophilia A treatment. FVIII-ΔII exhibits a 178 kDa single chain and a 91-87 kDa heavy-light chains dimer as measured by Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS). This method has also been chosen for a complete amino acids sequence verification and for glycosylation study of the recombinant protein. The copper ion identified by atomic absorption spectroscopy using Zeeman effect in each FVIII molecule is not directly related to FVIII activity but is a structural prerequisite for heavy-light chain association. The fluorescence maximum measured at 334 nm is consistent with Trp residues emission in a hydrophobic environment. This result is confirmed by kinetics of fluorescence emission which can be fitted by multiexponential decays characterizing a Trp in a folded protein. Fluorescence data also suggest that the two FVIII forms, which have the same specific activity, are similarly folded, in agreement with circular dichroïsm data. FVIII acts in the coagulation pathway in its activated form after cleavage by thrombin. This specific proteolysis leads to the separation of homologous FVIII domains which dissociate from the activated complex during the following inactivation phase. Spectroscopic data indicate that no significant structural modifications of the different FVIII domains occurs during the activation and inactivation process.

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1-52

IMMUNOSUPPRESSOR BINDING TO THE IMMUNOPHILIN FKBP59 AFFECTS THE LOCAL STRUCTURAL DYNAMICS OF A SURFACE β-STRAND: A TIME-RESOLVED FLUORESCENCE STUDY.

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The interaction of the immunophilin domain of FKBP59 (FKBP59-I) with immunosuppressant drugs was investigated by steady-state and time-resolved fluorescence of tryptophan. One of the two Trp residues present in this protein (W89), conserved in almost all immunophilins, is buried in the hydrophobic core and participates in the immunosuppressant binding. By comparison with the highly homologous protein FKBP12, containing only the buried Trp, we have concluded that its weak fluorescence is due to an atypical H-bond interaction involving the indole nitrogen and the Phe129 benzene ring. The second Trp residue (W59) in FKBP59-I is located on the external hydrophilic side of the 50-60 β-sheet (Craescu, C.T., Rouvière, N., Popescu, A., Cerpolini, E., Lebeau, M.-C., Baulieu, E.-E. and Mispelter, J., 1996, *Biochemistry 35, 11045-11052*) and is responsible for more than 95% of the fluorescence emission. The long lifetime of the major excited state, the large activation energy of thermal quenching and the rotational correlation time distribution pattern, suggest that its environment is not highly mobile. Binding of the immunosuppressant drugs FK506 and rapamycine leads to a ~60% decrease of the fluorescence intensity without any change in the fluorescence emission maximum. Time-resolved measurements show that this "quenching" is due to a conformational change which depletes the long excited state lifetime population to the profit of a more quenched minor excited state, which becomes prominent in the complexes. This is accompanied by a strong slowing down of the indole ring dynamics in the case of FK506 and by a complete immobilization in the case of rapamycine, as shown by two-dimensional (τ, θ) Maximum Entropy analysis of the polarized fluorescence decays. Binding of the immunosuppressant drugs therefore modifies the structure and the dynamics of the external side of the 50-60 β-sheet in FKBP59-I, which could be relevant for the formation of ternary complexes with other protein targets.

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1-53

VALUE OF RADIOPROTECTOR WR-2721 ON RADIATION INDUCED EYE DAMAGE .M. M. GAMAL[1],A. M. TALAAT[2] and L. K. HANAFY[3] .

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Many tools have been used to minimize radiation induced damage to vital organs. We assessed the efficacy of the radioprotector effects of WR-2721 in β-radiation induced eye damage. twenty New Zealand white rabbits were divided into radioprotected,radiounprotected and control groups.Eight rabbits treated with topical application of radioprotector WR-2721,after thirty minutes four rabbits received 2400 rads and four rabbits received 10,000 rads to their right eyes.Another four rabbits received 2400 rads and four rabbits received 10,000 rads to their right eyes without the use of radioprotector.After two months waiting period,the animals were sacrified and the change in protein and amino acids of both lens and aqueous humour were measured in both right (irradiated) and left (non-irradiated) eyes. Also,histopathological change of lenses and retina were determined.The radioprotective effects of WR-2721 vary with the amount of radiation used .In small doses of radiation,the change in protein and amino acids content of lens and aqueous humour is little in case of radiation protected eyes. Also,there is little histopathological changes. Although WR-2721 has been shown to be effective when administered systemically,its use has been limited by systemic side effects.Our study suggests a radioprotective effect of topical applied WR-2721 in radiation - induced cataract.

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1-54

COILED-COIL STRUCTURE FOR *neu*/c-*erb*-B2 TRANSMEMBRANE DOMAIN DIMERS. N. Garnier, N. Sajot, D. Genest and M. Genest.

The *neu*(rat)/c-*erb*-B2(human) proto-oncogenes encode proteins closely related to epidermal growth factor receptor which exhibit tyrosine kinase activity. As a general mechanism for transmembrane signal transduction, receptor activation results from dimerization process. Besides, dimerization and activity are dramatically increased by a point mutation within the transmembrane domain (TM) of the oncogenic products which bear a Glu residue in replacement of Val at the specific position neu_{664}/c-erb-$B2_{644}$. This activating amino acid substitution probably plays a significant role in transmembrane helix associations.

For a better understanding of specific interactions induced by the Glu mutation, computational searching for TM dimer models was undertaken. From a global search of all possible packing interactions between helices several crude models for dimeric association are predicted. It is shown that both wild and Glu mutated c-*erb*-B2 TM exhibit the same relative orientation for favourable interactions. Structure refinement using molecular dynamics techniques leads to left-handed coiled-coil structures. The mutation point belongs to the helix interface and models reveal inter helical hydrogen bonds involving the Glu side chains. Interestingly, *neu* TM dimer models evidence structure with similar left-handed helix winding stabilized by symmetric Glu hydrogen bonds.

These structural characteristics suggest a common mechanism for *neu*/c-*erb*-B2 TM dimerization.

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1-55

THE INFLUENCE OF GLU^{120} ON THE FLUORESCENCE OF TRP^{121} IN HUMAN CYCLOPHILIN A. M. Gastmans, Y. Engelborghs

Human cyclophilinA (hCypA) belongs to the family of peptidylprolyl cis-trans isomerases (PPIase) and it selectively binds the potent and clinically useful immunosuppressant cyclosporinA (CsA). Upon binding CsA, the PPIase activity of hCypA is abolished and there is an interesting twofold enhancement of the fluorescence of Trp^{121} in hCypA. Our goal is to search for an interpretation on the molecular level of this fluorescence enhancement and to characterize the fluorescence properties of Trp^{121}.

A study of the crystal and NMR structures of hCypA and its complex with CsA, revealed that one glutamate and two histidine residues can interact with Trp^{121} and change its fluorescence properties. The distance between the side chains of Glu^{120} and Trp^{121} in hCypA doubles upon binding CsA. If the resulting quenching interactions are pH dependent then it should reveal the pK_a of the interacting aminoacids. A study of the fluorescence intensity over the pH range 3-9, resulted in two sigmoidal curves with midpoints 4.2 and 5.7, values typically referring to glutamate and histidine. A pH titration could not be done on the complex of hCypA and CsA because of the dissociation of the complex at lower pH.

In a multifrequency phase fluorimetric study the fluorescence lifetimes of hCypA at pH8.0, pH3.7 and complexed with CsA (pH8.0) were determined. The fluorescence decay was well described by a sum of three exponentials. After complexation with CsA and after lowering the pH, the weihted average lifetime increases as expected.

Further investigation of the influence of Glu^{120} on the fluorescence of Trp^{121} will be done by mutagenesis.

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1-56

TEMPERATURE INDUCED CONFORMATIONAL TRANSITIONS IN FIBRINOGEN. S.V. Gatash and V.P. Berest.

In order to elucidate the conformational dynamic of fibrinogen molecule under physiological conditions the influence of the temperature (range of 4-50°C) on the bovine fibrinogen solution have been studied by using ultraviolet spectroscopy and very high frequency dielectrometry methods. The temperature dependences of the absorption intensity and first derivative peaks intensity of fibrinogen solution have been obtained. The changes of the intensity of maxima for derivative spectra of the fibrinogen solution have occured at the temperature : 8-12°C, 18-22°C and 35°C. This fact demonstrates the replacement of the polar groups in the protein molecule. The state of the water in solution has been studied because it is of importance for the changes of protein spatial organization. The temperature dependences of the real ε' and imaginary ε'' parts of complex permittivity $\varepsilon = \varepsilon' - j\,\varepsilon''$ of the fibrinogen solution have been measured. At the teperature 8-10°C, 20-22°C and 35°C the deviation from monotonousity of the temperature dependences of the ε' and ε'' of the fibrinogen solution has taken place. Such a deviation has not been observed in the case of pure solvent. This fact indicates the change of the ratio of the free and bound water in the system protein-water. Probably, the change of fibrinogen molecule conformation happens at these temperatures. Thus, besides known high and low temperature transitions the conformational transition at temperatures 20°C and 35°C have been observed in fibrinogen under physiological conditions.

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1-57

Folded and Partially Unfolded states of a Two Disulfide Derivative of Charybdotoxin J. Song, B. Gilquin, N. Jamin, E. Drakopoulou, M. Guenneugues, M. Dauplais, C. Vita, A. Ménez

The α/ß scorpion fold consisting of a short α-helix and ß-sheet is a structural motif common to scorpion toxins, insect defensins and plant γ-thionins that invariably contains three disulfides. CHABII is a two disulfide derivative of the scorpion toxin charybdotoxin (ChTX), chemically synthesized by inserting two (L)-α-aminobutyric acid in place of the two half-cystine residues involved in the disulfide 13-33. This disulfide is one of the two disulfides which connect the α-helix to the ß-sheet. The solution structure of CHABII was determined at pH 6.3 and 5 °C using 2D NMR and simulated annealing. The fold of CHABII is similar to that of ChTX as indicated by the low value of the averaged backbone atomic rms deviation between the two protein structures (1.44 Å). The packing of the hydrophobic core is well-preserved underlying the critical structural role of the hydrophobic interactions even for such a small and cysteine-rich protein as ChTX.

Analysis of NOESY maps at pH 6.0, 5.5, 4.6 and 4.0 at 5° reveals that NOE effects of secondary structure and of tertiary topology, disappear in a stepwise manner with lowering pH. However, at pH 4.0, most of the remaining long range NOE effects belong to the residues of the hydrophobic core. This persistence of the native like packing core at 5° and pH 4.0 make evidence of the important role of the hydrophobic core in the stability of this a/ß motif.

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1-58

CHARACTERIZATION OF THE WARFARIN BINDING SITE ON HUMAN SERUM ALBUMIN BY THE PRODAN FLUORESCENT PROBE F. MORENO and J.GONZALEZ-JIMENEZ

The compound 6-propionyl-2-(N,N-dimethyl)-aminonaphthalene, (PRODAN), is an efficient fluorescent probe for proteins. PRODAN in aqueous solution has an emission maximun at 530 nm, but when it is bound to human serum albumin (HSA) the peak shifts to 485 nm. Assuming that the degree of association to be proportional to the 485 intensity peak, PRODAN is found to bind to a single site on HSA with a high affinity. The association constant at 25°C was stimated as $3.9x10^5$ by the same methos previously used with other drugs-HSA interactions (J. González-Jiménez et al. J.Pharm. Pharmacol, 47, 1995) Attempe were made to find if this probe could be used as a label of a specific binding site on HSA.Bound PRODAN can be displaced from HSA by warfarin which binds to the site I on HSA with an association constant of $2.5x10^6$. The emission spectra obtained by adding warfarin to HSA labeled with PRODAN in aqueous buffer, showed a decrease in the intensity of the PRODAN-HSA emission band located at 485 nm and concomitantly an increase in the emission band relative to the free PRODAN located at 525 nm.

Furthermore, Warfarin has the advantage that in water, shows a weak fluorescence at 365 nm by exciting at 320 nm, but bound to HSA the fluorescence intensity is highly enhanced. On binding of warfarin to HSA labeled with PRODAN, the fluorescence polarization values increased (excitation= 320 nm, emission=365) as a consequence of its rotational impediment A concomitant diminution of the PRODAN-HSA fluorescence intensity at 485nm was also observed. So, there are two evidences to support that PRONDAN binds at the same site that warfarin in HSA and therefore, PRODAN can be used as a fluorescent probe to characterize the warfarin binding site on the HSA, or Site I. It may also be a usseful procedure to follow drug displacements.

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1-59

A NEW TECHNIQUE TO QUANTIFY PROTEIN CONFORMATIONAL TRANSITIONS IN SOLUTION. *E. Gräf[1], P. Bugnon[2], A.E. Merbach[2], M. Doludda[1], E. Schick[1], E. Lewitzki[1] and H. Ruf[1].*

Although the concept of functionally relevant protein conformational transitions occurring subsequent to an usually fast and selective binding step is generally used in biological sciences there is undoubtebly a lack of methods allowing to quantify such transition in solution. There are no means to decide whether a transition involves a large or only a small local rearrangement. Conformational transitions in terms of a single reaction step can be resolved by employing fast chemical reaction techniques such as the stopped-flow method. The determination of the rate constants of a transition as a function of pressure up to 2000 bar can allow the determination of the volume change related to the conformational transition together with the two activation volumes and thus allows to quantify the transition in terms of these parameters.

This is realized for the selective binding of K^+ to Na,K-ATPase by carrying out thermodynamic and kinetic studies. The binding of two K^+ in the µsec time range is followed by a conformational transition, which is assigned to alkali ion occlusion and characterized by a forward and backward rate constant around 500 and 1 sec^{-1} at ambient pressure and a volume change of -32 ml mol^{-1}. The activation volume of the forward and backward conformational transition is +39 and +71 ml mol^{-1}. These activation volumes are unusually high and indicate that the volume of the transition state of the conformational change is considerably larger than that of the related two equilibrium states before and after the transition. Because a small local conformational change upon K^+ binding is likely to be characterized by smaller activation volumes, the obeserved transition is thus attributed to the rearrangement of a segment of the protein. This conformational transition is linked to active K^+ transport.

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1-60

NATIVE AND NON-NATIVE CAPPING BOXES AS POSSIBLE FOLDING PATHWAY DETERMINANTS IN HELICAL PROTEINS: AN EXAMPLE FROM ANNEXIN FRAGMENTS. R. Guerois, F. Baleux[1], T. Huynh-Dinh[1], J.-M. Neumann & A. Sanson.

Protein fragments, which have lost all or part of their long range interactions leading normally to a cooperative folding, can be used as pertinent indicators of the local early state of the protein folding. We are currently using this approach to study the folding of Annexins. Here, we will focus on the (A) fragment spanning the A helix of Annexin I domaine 2. This fragment indeed includes several interesting features whose study could help characterizing some aspects of the folding pathway of the protein:

L K T P A Q F D A D E L R A A M K G

In particular, two potential capping boxes 'T3xxQ6' and 'D8xxE11'. The former corresponds to the *native* hydrogen bonds network stabilizing the A helix amino end. The latter is a *non-native* capping box which breaks the helix at residue D8.

In aqueous solution, using 2D NMR techniques, several populations of conformers can be distinguished. The first one presents a helical conformation from residue A5 to M16. The other one is also helical, but the helix is broken at residue D8. A quantitative analysis of those conformations has been carried out. The importance of each local structure in the stability of helical conformations was achieved by the study of eight punctual mutants.

The non-native 'D8xxE11' capping box, which is of course dismantled in the final step of the protein folding, involves highly conserved residues among the annexin family. We postulate that the double capping signal present in the A helix may play an important role in the folding pathways and kinetics.

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1-61

LOCALIZATION OF CARBONIC ANHYDRASE IN THE LEAVES OF AMARANT. N.M. Guliev, H.G. Babayev, Sh.M. Bayramov and J.A. Aliev.

Carbonic anhydrase (carbonate-hydro-lyase; EC 4.2.1.1) catalysing the reversible hydration of CO_2 in C_4 plants has not been thoroughly studied. For the study of physiological functions of carbonic anhydrase in C_4 plants the problem regarding intracellular localisation of this enzyme in these plants is of special importance. In the study of localisation of carbonic anhydrase in the leaves of C_4 plant of amaranth (Amaranthus cruentus L.), 40-45 daily plants have been used. With the help of methods elaborated by us we obtained the intracellular fractions of mesophyll and bundle sheath cells. The purity of obtained fractions has been tested by microscopic methods and by determination of the activity of marker enzymes. The results obtained testify that the principal part of carbonic anhydrase activity is found out in the soluble fraction of cytoplasm of mesophyll cells and in thylakoid membranes of chloroplasts of bundle sheath cells. These enzyme forms differ by physico-chemical parameters and carry out different physiological functions.

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1-62

BINDING KINETICS OF AN SH2-DOMAIN MONITORED BY NMR SPECTROSCOPY. U. Günther[1], B. Schaffhausen[2], H. Rüterjans[1]

SH2 domains are key protein modules in cellular regulation. SH2s respond to tyrosine phosphorylation by binding tyrosine-phosphorylated sequences. Structures of different SH2 domains exhibit a central β-sheet which is flanked by two α-helices. Binding of phosphopeptides was first described as a prong-and-socket model. In the case of the N-SH2 domain of the p85 subunit of PI-3'-kinase phosphopeptide binding causes a rearrangement in a loop (BG) which is reflected in a significant chemical shift change of the ^{15}N amide signal of tyr416 in the BG loop.
By comparing chemical shifts of amide protons in different phosphopeptide complexes we determined the role of individual amino acids in the protein and in the phosphopeptide for specific interaction. By simulation of NMR lineshapes in 2D-NMR spectra recorded during phosphopeptide titration we have determined kinetic parameters for individual amino acids of the protein. This analysis shows that the life time of the protein-peptide interaction varies for different positions of the protein. The binding reaction follows a second order kinetic for all amino acids. In addition we observe a two-step binding mechanism for some amino acids. This may be correlated to the rearrangement upon binding in the BG loop.
Examination of substituted ligand peptides demonstrates interactions among different binding regions of the protein. This kind of NMR chemical shift and kinetic analysis, comparing different ligands, should provide a useful root for the development of protein inhibitors.

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1-63

MODELBUILDING OF MULTIMERIC PROTEINS FROM SMALL-ANGLE X-RAY DATA WITH PARTIALLY KNOWN X-RAY STRUCTURES. H. Hartmann, B. Lohkamp and H. Decker
X-ray crystallography of proteins yields a precision for the atomic coordinates of about 1/10 of the experimental resolution, because the structure of the building blocks, the amino acids, is known in great detail. In a similar manner the effective resolution of methods with an inherent low resolution like electron microscopy or small-angle scattering of X-rays or neutrons can be improved, when the X-ray structures of some or all of the constituents of a complex molecule like the multimeric arthropod hemocyanins are available. We used this method to model the structure of the 4x6-meric hemocyanin from the tarantula *Eurypelma californicum* in the oxy and deoxy state from small-angle X-ray scattering data. As a building block the X-ray structure of the single homohexamer of *Limulus polyphemus* was used. The fit to the experimental data reveals, that in the deoxy state the 4 hexamers are arranged in a rectangular geometry and are nearly in plane with a center to center distance of 10.3nm within the 12-meric halfmolecule and of 10.4nm between the two identical 12-mers. In contrast, the fully oxygenated molecule has a rhomboid distortion. The halfmolecules are shifted against each other by 1.6±0.4nm and tilted out of plane by about $\pm 12^0$. This is governed by a decrease of the relative rotation of the hexamers within the halfmolecule from 125^0 for the dexoxy to 110^0 in the oxy structure. The great structural rearrangement takes place without disrupting the strong interaction between the halfmolecules caused by the b and c subunits.

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1-64

THE ROLE OF TROPONIN C CONFORMATIONAL CHANGES IN THE MUSCLE CONTRACTION REGULATION. N. Miroshnichenko and D. Havrilev
Troponin C (TnC) mediates regulation of skeletal muscle contractile activity responding to the local changes of Ca^{2+} ions concentration. So far there is no adequate model of the muscle contraction regulation. Our investigation of the three-dimensional structure of sarcomere led us to a conclusion that TnC cannot sterically block the interaction of myosin heads with actin thin filament. Physical-chemical analyses (molecular modeling, computer graphics, influence of the changes of pH and Ca^{2+} ions concentrations) shown that the interaction of Ca ions with Tn C is able to cause only small structural changes in N-domain of this molecule. The constructed TnC model demonstrate that more than six amino acids take part in the Ca^{2+}-coordination in both sites I and II. The formation of TnC N-domain structure with Ca^{2+} ions leads to the distortion of the short antiparallel β-structure between sites I and II. It was found that even small increases of Ca^{2+} concentration (up to $10^{-7}M$) change TnC fluorescence. It was shown that the level of fluorescence of Tn C is restored if Ca^{2+} concentration had gradually decreased from $10^{-6}M$ down to $5*10^{-7}M$. A new concept of a muscle contraction regulation is being elaborated. It is suggested that not all thin filament as well as not all Tn C but only the parts of this supramolecular structure that are located at this ends of the thin filaments participate in a muscle contraction regulation.

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1-65

CONFORMATIONAL STUDY OF PEPTIDES ADDRESSING INTRACELLULAR DOMAINS. A. Heitz, L. Chaloin, P. Vidal and F. Heitz.
Two series of peptides built of the association of a nuclear localization sequence (NLS) with either a signal peptide or a sequence issued from the fusion protein gp41 of HIV1 (AcMGLGLHLLVLAAALQGAWSQPKKKRKVCya [1] AcGALFLGWLGAAGSTMGAWSQPKKKRKVCya [2] respectively) are shown to localize spontaneously the intracellular domains with a final localization which is mainly nuclear. In order to elucidate the membrane translocation mecanism we made a conformational study of these peptides in solution and in membrane mimicking environments. On the basis of CD, IR and NMR spectroscopy it was shown that all peptides adopt a random coil form in pure water but that they are largely in an α helical conformation when incorporated in sodium dodecyl sulfate (SDS) or dodecylphosphocholine (DPC) micelles. NMR study in SDS-d25 showed that the helical domain correspond to the hydrophobic part of the peptides while the hydrophilic one which correpond to the NLS sequence remains unordered. Fluorescence measurements carried out on the gp41 derived peptide [2] which contains a tryptophane residue in position 7 of the hydrophobic sequence show that when interacting with SDS, the fluorescence is strongly blue shifted (from 354 to 330 nm). This indicates that this sequence is embedded in the hydrophobic domain of the micelles.

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1-66

TIME RESOLVED FT-IR AND FT-RAMAN STUDIES OF THE M HETEROGENEITY IN THE BACTERIORHODOPSIN PHOTOCYCLE. B. Heßling and K. Gerwert.

The reprotonation of the transiently deprotonated Schiff base is the crucial step in the photocycle of Bacteriorhodopsin. After proton transfer to the internal proton acceptor D85, which is deprotonated in the unphotolysed state, the Schiff base regains a proton from D96 which is located on the cytoplasmic side of the membrane (1,2). Many authors assume that at least two M substates exist which differ in the proton accessibility of the Schiff base: An early M_1 intermediate whose Schiff base is still orientated towards D85 and a late M_2 intermediate which has turned its Schiff base accessibility to the internal proton donor D96.

We utilise the M back photoreaction in order to clarify the molecular nature of the reprotonation process. Double flash experiments in combination with nanosecond step-scan FT-IR spectroscopy attribute the orientational change of the Schiff base to a conformational change in the protein's backbone (3). The experimental results argue against the involvement of a secondary isomerisation in the polyene chain during the M_1 to M_2 transition. After the second pulse induced photoisomerisation, the Schiff base regains a proton from the former proton acceptor D85. This result follows from the simultaneous disappearance of the carbonyl band at 1762 cm^{-1} and the reappearance of the ethylenic vibration at 1527cm^{-1}.

FT-Raman spectroscopy, which monitors primarily vibrations of the chromophore, reveals no spectral evidence for the different M substates in the photocycle of the wild-type. However, when the proton donor D96 is replaced by an asparagine a spectrally distinct M substate with a blue-shifted ethylenic frequency appears. This is consistent with the observed shift of the visible absorption maximum in D96N. Moreover, the mutant gives no indication of any change in the C-C stretch vibration region in M that characterises the isomerisation of the polyene.

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(3) Heßling, B., R. Rammelsberg and K. Gerwert, submitted

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1-67

EFFECT OF CATIONS ON PEPTIDES: A CD AND FTIR SPECTROSCOPIC STUDY. Zs. Majer, E. Vass, L. Somogyi and M. Hollósi.

Cations play an important role in the biological function of proteins and peptides. The toxicity of certain cations originates in replacing vital ones due to stronger binding to "functional sites" or inducing unfavourable conformational changes of the peptide skeleton. This paper reports systematic studies of the effect of Al^{3+}, Ca^{2+}, Mg^{2+} and Na^{2+} ions on peptide fragments of the β-amyloid protein and the microtubule associated protein, tau. Circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopy was used to monitor changes of the backbone conformation in terms of the structure of the peptide (absence or presence and relative position of acidic, basic or alcoholic side-chain functionalities). Based on CD studies, cation binding in water does not have a significant effect upon backbone conformation. Using trifluoroethanol (TFE), a solvent of decreased dielectric constant, cation-dependent changes of the CD spectra were observed. Resolution-enhanced FTIR spectra in TFE showed shifts of the ratio of open and folded (β- and/or γ-turn) conformers. Cation binding to skeletal amide group(s) resulted in low frequency (1620–1600 cm^{-1}) amide I component bands. FTIR spectroscopy also reflected ligand preference of the cation. In general, multifunctional peptides suffered less expressed changes of the skeletal conformation than peptides lacking COOH or OH functional groups. Aluminum was found to induce more profound conformational changes than the other cations used. CD spectroscopy is a simple tool for probing cation selectivity of peptides and proteins and for determining stoichiometry and cation binding constant.

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1-68

INTERACTIONS OF CYTOCHROME P-450 2B4 WITH PROTEIN REDOX PARTNERS STUDIED BY HIGH PRESSURE SPECTROFLUORIMETRY. G. Hui Bon Hoa and D. Davydov.

The aim of the present study is to probe the role of surface charges and surface-bound water molecules in the formation of the complexes of the rabbit liver microsomal cytochrome P-450 2B4 with NADPH-cytochrome P-450 reductase (NCPR) and cytochrome b5. We have developped direct methods to follow these interactions. NCPR was labelled with fluorescent probes 7-ethyamino-3-(4'-maleimidilphenyl)-4-methylcoumarinmaleimide (CPM) and Zn-protoporphyrin(IX)-substituted derivative of cytochrome b5 was used. Binding affinities were determined by following the quenching of the fluorescence-proteins upon formation of 1:1 complex with cytochrome P-450 2B4. Hydrostatic pressure (P) was used as a perturbant tool to shift the reaction equilibrium allowing the determination of their reaction volumes. The results of the binding studies showed that the formation of both complexes follows the behavior of a simple bimolecular association with K_d = 0.038 μM and k_{on} = 6.5 10^5 $M^{-1}s^{-1}$ for 2B4-NCPRcpm complex and K_d = 1.2 μM for 2B4-Zn-b5 complex (both at I=58 mM). An increase in ionic strength (I) induced a dissociation of both complexes. 2B4-NCPRcpm complex was dissociated by an increase of pressure, the obtained volume (ΔV^*_d = -65 ml/mol) was compatible with a model of charge-driven protein interactions. In the contrary pressure favored the association of 2B4-Zn-b5 couple. The dissociation volume changes (ΔV^*_d) of this complex was dependent on ionic strength: it increases from 29 to 67 ml/mol when I decreases from 30 to 6 mM. The formation of this complex seems to be accompanied by trapping of several water molecules resulting presumably from the cleavage of some internal salt bridges in the molecules.

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EPR SPECTROSCOPY OF THE REDOX ACTIVE CENTRES IN QUINOLINE-2-, ISOQUINOLINE-1-OXIDOREDUCTASE AND QUINALDINE-4-OXIDASE. C. Canne, I. Stephan, J. Finsterbusch, R. Kappl, S. Fetzner, F. Lingens and J. Hüttermann.

Three bacterial enzymes of the xanthine oxidase family, containing molybdopterin dinucleotide cofactors, were investigated by EPR spectroscopy to elucidate mechanistical aspects of the specific substrate hydroxylation and to gain structural information: quinoline-2-oxidoreductase (QuinOR), quinaldine-4-oxidase (QualOX) (>300 KDa, substructure $\alpha_2\beta_2\gamma_2$) and isoquinoline-1-oxidoreductase (IsoOR), a heterodimer of 95 KDa. In QuinOR and QualOX besides two distinct [2Fe-2S] centres the signals of two differing FAD radical species (the neutral and anionic form) and of several Mo(V)-centered species appear after reduction with substrates or dithionite. In IsoOR, lacking the FAD cofactor, no free radical signal typical for the flavin centered radical species is observed. In this enzyme one of the two distinct [2Fe-2S] centres exhibits a well resolved line splitting depending on the reduction state of the other iron sulfur centre. It is independent on the excitation frequency and therefore correlated to a magnetic dipolar interaction between both [2Fe-2S] centres, from which a distance of ca. 15 Å is estimated. In addition, the three enzymes show pronounced differences in the redox potentials of their [2Fe-2S] centres, as determined by titration with dithionite (QuinOR: -155/-195 mV, QualOX: -70/-250 mV, IsoOR: +10/+65 mV), which may be indicative for a different structural environment and possibly different functional roles of the iron-sulfur clusters in the enzyme turnover. The findings are compared to other well characterized enzymes like xanthine oxidase.

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1-70

THE 3D STRUCTURE OF THE COMPLEX BETWEEN THE ANTI-AChR Fv ANTIBODY FRAGMENT AND THE [G^{70},Nle^{76}]MIR DECAPEPTIDE ANTIGEN. Jens Kleinjung, Marie-Christine Petit, Piotr Orlewski, Avgi Mamalaki, Christos Liolitsas, Sokrates J. Tzartos, Vassilios Tsikaris, Maria Sakarellos-Daitsiotis, Constantinos Sakarellos, and Manh-Thong Cung.

Monoclonal antibodies (mAbs) raised against the main immunogenic region (MIR) of the acetylcholine receptor (AChR) are potent modulators of the antigenic reaction of AChR in animals and human cell cultures. The epitope of AChR involved in the binding of mAbs has been localized between residues 67-76 of the AChR α-subunit. A modified MIR sequence of *Torpedo* electric organ, the decapeptide W^{67}NPG^{70}DYGGINle^{76}, has been synthesized by solid phase synthesis. Elucidation of the structure of the [G^{70},Nle^{76}]MIR decapeptide complexed to the anti-MIR mAb in solution (molar ratio 50/1) has been performed using transferred NOE experiments. Analysis of the spectra gave distance constraints, which lead to a well defined solution structure. However, modelling of antibody-antigen affinity requires information on both binding sites. Therefore, a 3D model of the single-chain Fv fragment (scFv) of the anti-MIR mAb has been constructed by homology modelling. Molecular docking was initialized by parallel and independent fit onto both structures, the modelled binding site of the antibody and the [G^{70},NLE^{76}]MIR decapeptide derived from NMR experiments.

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1-71

INTERACTION OF INFLUENZA HEMAGGLUTININ WITH NEUTRALISING ANTIBODIES. D. Fleury(1), T. Bizebard(1), J.J. Skehel(2) and M. Knossow(1).

Influenza hemagglutinin is the major viral surface glycoprotein; it has two functions in infection, receptor binding and membrane fusion. Antibodies that recognize HA block virus infection and, probably as a consequence, influenza antigenic drift is accompanied by changes in HA amino acid sequence. The crystal structures of a number of HA-antibody complexes have been determined and provide definitive proof of the sites of antibody binding on HA. Antibodies bound near the receptor binding site, such as antibody HC19, inhibit receptor binding by competing for this position on HA. Antibodies binding at sites outside the receptor pocket appear to prevent receptor binding less directly by covering the pocket on the basis of their proximity to it or their size.

Escape to neutralization of infectivity is ensured by mutations of HA at residues buried in the HA-antibody interface. In the case of antibodies such as HC19 whose interface with HA comprises the receptor binding site, escape mutations occur at positions outside the receptor binding site. The conservation of the receptor binding site is made possible even under the selective pressure of antibodies binding to this region of HA because the greater surface area of an antibody footprint by comparison with the surface area of the receptor binding site results in all antibodies interfaces containing residues not required for the receptor binding function; mutations of these residues yield mutants that escape infectivity neutralisation.

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1-72

DYNAMICS OF PROTEINS DESCRIBED BY DIELECTRIC RELAXATION MODELS. MOESSBAUER SPECTROSCOPY (MS) AND RAYLEIGH SCATTERING OF MOESSBAUER RADIATION (RSMR) DATA. Yu. Krupyanskii, A.Moroz, I.Cheng and F. Parak.

Protein specific modes of motions are found in myoglobin crystals (by MS) and in hydrated human serum albumen (HSA) (by RSMR) above 180-200 K. In this contribution we show that this type of motions can be analyzed in analogy to dielectric relaxation in glasses and supercooled liquids by a Davidson -Cole, a Cole-Cole or a Havriliak-Negami distribution. The observed temperature dependencies of the line shape of Moessbauer and RSMR spectra can be described within the framework of abovementioned non-Debye models. However, the temperature dependence of the obtained parameters is quite unusual indicating a broadening of a distributions with the increase of a temperature instead of motional narrowing which one can expect taking into account data for the rebinding kinetics (and there interpretation[1]) and relaxation in proteins (protein quakes and pressure jump studies). One can assume that more and more conformational substates become accessible with increasing temperature. The obtained values of activation energy and pre-exponential factor of relaxation time τ_c deduced from the Moessbauer spectroscopy study of myoglobin are rather typical for β-relaxation. The same values for τ_c deduced from the RSMR study of HSA at T≤280 K are typical for α-relaxation and in the temperature region 280-310 K for αβ-relaxation. The obtained results shows that the well-established at T<200K analogy between protein and glasses (glassforming liquids) should not been exaggerated at T>200 K.

[1] Zharikov A.A,.Fisher S.F. (1996) Scaling law for the non-exponential ligand rebinding of CO in myoglobin.Chem.Phys. Letters. 263:749-758

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1-73

INVESTIGATION OF COMPACT NON-NATIVE STATES OF THE GLOBULAR PROTEINS BY ^{1}H NMR. V.P. Kutyshenko and L.S. Kivaeva.

The molten globule state of α-lactalbumin and carboanhydrase B were studied by one-dimensional high resolution NMR spectroscopy. The spectra of any protein in this state are low informative because of the absence of the chemical shifts dispersion. The spin diffusion method that we originally designed (Yu.V.Griko and V.P.Kutyshenko, Biophys.J., 67, 356-363,1994) allows us to obtain precise information about molecules' compactness.

The experiments at different temperature conditions show the existence of high-ordered regions in the molten globule state of the α-lactalbumine. The theoretical analysis of the relaxation experimental data confirmed the presence of some few protein conformations. Although the protein molecule is rather structured and compacted it would be possible to be in each different conformations at certain periods of time. However, the presence of a very stable conformation, totally different from the native one, is also possible, for some parts of the polipeptide chain.

Our investigations confirmed that the molten globule state of α-lactalbumine (pH 2) and carboanhydrase B (pH 4) is a true thermodynamic state and the transition to the unfolded form is a first order phase transition.

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1-74

CONFORMATIONAL CONSEQUENCES OF THE PHOSPHORYLATION OF THE T-CELL RECEPTOR COMPLEX ζ-CHAIN. I. Laczkó, M. Hollósi, É. Monostori, G.K.Tóth.

The ζ-chain of the T-cell receptor (TCR)/CD3 complex plays a central role in cell signalling via the TCR. Following receptor stimulation, the ζ-chain is phosphorylated on tyrosine residues in certain positions in the intracellular region of this molecule. However, it has not yet been revealed which of the tyrosines are phosphorylated.

In order to study if the phosphorylation of the shorter and longer fragments of ζ-chain causes comparable conformational changes, the following short peptide fragments (P69, P80, P106, P119, P138, P149), three longer fragments containing the P69+P80 (ζa), P106+P119 (ζb), P138+P149 (ζc) sequences and the corresponding phosphorylated derivatives were synthesized by solid-phase methodology:

P69 P80 P138 P149
ζ(a): ***NQLYNEL***NLGR***REEYDVL,*** ζ(c) ***DGLYQGLS***TAT***KDTYDAL,***

P106 P119
ζ(b): ***QEGLYNEL***QKDKM***AEAYSEIG***

The secondary structures of peptides were studied by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy in water and trifluoroethanol (TFE) solutions. The non-phosphorylated short peptides are mainly present as a β-turn/unordered conformer mixture in TFE. This conformational equilibrium is shifted in most cases towards the β-sheet structure upon phosphorylation. Selective phosphorylation of one or the other tyrosine of the longer peptides results in the predominance of α-helical conformation. However, a substantial amount of β-sheet structure is formed phosphorylated the peptides on both tyrosines.

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1-75

SOLUTION STRUCTURE OF DROSOMYCINE, AN INSECT POTENT ANTIFUNGAL PROTEIN .

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In response to a septic injury, larvae and adults of *Drosophila* produce a considerable amount of drosomycin, a 44-residue protein containing 4 disulfide bridges. This protein, inactive against bacteria, exhibits potent antifungal activity, and shows significant homologies with plant antifungal proteins isolated from the seeds of radish. Its three-dimensional solution structure was established from two-dimensional NMR data and molecular modeling. Its fold involves a helix fastened to a three stranded beta-sheet by two disulfide bridges defining a CSαβ motif. This motif was previously described in insect defensin, plant defensin, γ-thionin and scorpion toxin structures. Comparison of high resolution solution structures of drosomycin with γ-thionin structures reveals the structural importance of some residues such as Ser4, Tyr7, Gly9, Gly31 and the probable functional importance of Glu26 and Lys38. The use of programm 'cavité' which aims at delineating cavities in protein structures, revealed the presence of a hydrophobic pocket of moderate dimensions and compatible with a water molecule size, inside the protein core. This solvent molecule should be important to the protein three dimensional structure stability.

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1-76

A NEW CONCEPT TO EXPLAIN THE RESISTANCE OF A THERMOPHILIC PROTEIN TO HEAT, COLD and PRESSURE.

E. Mombelli[a], P. Tortora[b], M. Afshar[c], N. Bec[a] and R. Lange[a]

Ribonuclease P2 (7 kDa) from *S. solfataricus* is extremely resistant to pressure and heat. This protein has a large hydrophilic mantle and a hydrophobic core which contains three aromatic residues (Phe5, Phe31 and Tyr33). A molecular dynamics simulation reveals that these residues are arranged in a fish-bone geometry and have strong van der Waals interaction energies. For an understanding of the structural basis of its stability, we studied the thermodynamics of the heat, cold and pressure induced protein conformational changes of the wild type and of the F31A and F31Y mutants, by analyzing the protein UV absorbance in the fourth derivative mode. By this method we probed the properties of the hydrophobic aromatic core. Heat and cold denaturation of both mutants, as well as denaturation by pressure of the F31A mutant led to strong blue shifts of the derivative spectrum, indicating increased solvent exposure of Tyr33. For the F31Y mutant, high pressure protected the protein against thermal denaturation. In contrast, the hydration of the core of the wild type protein did not change under extreme conditions of temperature (-20 to +90°C) and pressure (500MPa). In comparison, a much lower stability and shallow transitions had been observed previously for the overall protein structure (Knapp et al., 1996, J. Mol. Biol., 264, 1132). The response of P2 to extreme conditions may be explained by a gradual multi-stage unfolding mechanism of larger peripheral parts of the protein. However, the melting is not complete, since the outer parts are linked to a very stable, stone-like hydrophobic core, which is stabilized by particular aromatic interactions.

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1-77

STABILITY OF THE DIMERIC PROAEROLYSIN AND THE AEROLYSIN HEPTAMER.

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Proaerolysin is secreted by *Aeromonas hydrophila* as a soluble dimeric protoxin. C-terminal cleavage leads to activation of the toxin. Heptamerization and pore formation then occur inducing cell lysis. Transition from dimer to heptamer certainly involves folding/unfolding processes. To decipher this mechanism, we have analyzed the unfolding of dimeric proaerolysin and heptameric aerolysin in urea and GuHCl. The results showed that proaerolysin unfolds in two steps and that the two disulfides bridges significantly contribute to the stability of the protein. By disturbing the bridges either with DTT or by mutagenesis, we could show that the elongated C-terminal domain unfolds first. Therefore, the N-terminal domain appears to be the most stable part of the protoxin, and perhaps maintains the two monomers together. The aerolysin heptamer remained assembled and fully folded even in 8 M urea illustrating its uncommon stability. Partial unfolding could be observed in GuHCl. To investigate which part of the protein maintains the heptamer assembled, we have searched for limited digests that did not affect assembly. A 200Kda complex was obtained after treatment of the heptamer in SDS with trypsin. N-terminal sequencing showed that the N-terminal domain had been removed. This truncated oligomer still migrated as a complex on SDS gels, indicating that it does not require the N-terminal domain to remain assembled. In conclusion, the N-terminal domain of proaerolysin is the most stable part of the protoxin but it is not required to maintain the heptamer assembled.

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1-78

INVESTIGATIONS OF AN OLIGOMERIC PROTEIN FOLDING. C. Leydier, E. Clottes, F. Couthon and C. Vial.

The folding mechanism of homodimeric MM creatine kinase (MM-CK) was investigated by kinetic and equilibrium experiments using activity, spectroscopic and hydrodynamic measurements and chemical modifications.

The equilibrium denaturation of MM-CK induced by guanidinium chloride (GdnHCl) occurs in two steps indicating the existence of two thermodynamically stable intermediate states. The first one (between 0.6 and 1 M GdnHCl) exhibits the same features as a molten globule (Rs=3.7 nm, λmax=342 nm, 2/4 Trp and 2-3/4 Cys accessible per monomer). The second intermediate state (between 1.2 and 1.6 M GdnHCl), which is less organized than the molten globule species, may correspond to a pre-molten globule state (Rs=4.8 nm, λmax=345 nm, 2/4 Trp and 3-4/4 Cys accessible per monomer).

The refolding kinetics induced by a ten-fold dilution from 3 M GdnHCl indicate that the protein refolds through a multistep process. During the dead-time of mixing, hydrophobic collapse leads to a partially folded state which rapidly goes through a transient monomeric intermediate conformation (Rs=3.3 nm, λmax=339 nm, 1/4 Cys accessible per monomer). One part (45%) of this transient monomeric state dimerizes to give an active enzyme (Rs=3.8 nm, λmax=333nm, 1/4 Trp and 1/4 Cys accessible). However, possibly due to proline isomerization, a large part of it is trapped into a non-productive, non-dimerizable conformation that limits the extent of renaturation. The control of the amount of the latter species may determine the correct and rapid folding of this dimeric protein.

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1-79

CHARACTERIZATION OF STRUCTURAL TRANSITIONS IN ARTHROPOD HEMOCYANINS BY SMALL-ANGLE X-RAY SCATTERING (SAXS). B. Lohkamp, H. Hartmann and H. Decker

Hemocyanins are oxygen transport proteins of invertebrates. The hemocyanins from arthropods consist of 6 to 8x6 monomers with a binuclear oxygen binding Cu-center per subunit. They reveal a high cooperativity, which is controlled by pH and a variety of metabolites. But the molecular mechanism is not known up to now. The X-ray structures of an oxygenated and deoxygenated 6-meric hemocyanin show no significant structural differences, which could explain the cooperativity. It has been shown, however, that the structure of these large proteins in a rigid environment could be rather different compared to their conformation in solution (Decker et al. 1996). We measured the small-angle X-ray scattering of dilute (6mg/ml) solutions of the 2x6-meric hemocyanin from *Homarus americanus* in the oxygenated and deoxygenated states at two pH-values and the influence of the effector molecule lactate. The measured scattering curves are interpreted with atomic models based on the X-ray structure of a 6-meric hemocyanin. The results show that the overall dimensions of the fully oxygenated conformation (pH 8.4) are smaller by about 0.5nm compared to the deoxy structure. At low pH 7.2 and oxygenating conditions the conformation of the molecule is somewhere between the oxy and deoxy conformation. Addition of lactate shifts the equilibrium of conformations back to the oxy structure. These results are in agreement with oxygen-binding studies and predictions of the nesting model.

Decker H, Hartmann H, Sterner R, Schwarz E, Pilz I (1996) Small-angle X-ray scattering reveals differences between the quaternary structures of oxygenated and deoxygenated tarantula hemocyanin. FEBS Letters 393:226-230

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1-80

INTERNAL DYNAMICS OF MYOSIN HEADS IN MUSCLE FIBRES BY DSC AND EPR. D. Lőrinczy, F. Könczöl and J. Belagyi

Conventional and saturation transfer electron paramagnetic resonance spectroscopy (EPR and ST EPR) and differential scanning calorimetry (DSC) were used to study the internal dynamics and thermal unfolding of myosin heads in muscle fibre and in myosin solution. Experiments were performed on chemically skinned fibres from m. psoas of rabbit and on cardic myosin isolated from bovine heart muscle in different states as rigor, ADP-state (strongly binding state) and ATP.V_i (weakly binding state).

Muscle fibres were spin-labelled with an isothiocyanate-based probe molecule at the reactive sulfhydryl sites (Cys-707) of the motor domain. In the presence of nucleotides, ADP or the nonhydrolyzable analogue of ATP, AMP.PNP and ADP or ATP plus orthovanadate, the conventional EPR spectra showed large changes in the ordering of the probe molecules in fibres, and a new distribution appeared. (ADP+orthovanadate) and AMP.PNP increased the orientational disorder of myosin heads; a random population was superimposed on the ADP-like spectrum evidencing conformational and motional changes in the internal structure of myosin heads. The fraction of ordered population depended on the nucleotide, when ADP was substituted by ATP, only the random population was detected. Our results on AMP.PNP fibres suggest that about half of the heads represents a disordered population with reduced rate of rotational motion as reported earlier by Fajer and coworkers (*Biophys. J.* **53** 513, 1988) and Marston and coworkers (*J.Mol. Biol.* **104** 263, 1976), However, spectrum analysis supports that the myosin heads which exhibit high degree of order are in strongly binding state, the orientation of heads being attached to actin differ from that of rigor.

Thermal unfolding in rigor is composed from a low temperature transition which might be attributed to the interaction of LC-2 light chain with the 20 kDa domain of myosin head, the two higher temperature meltings refer to the head region of myosin. DSC measurements support the view that addition of nucleotides produces conformational changes in the multisubunit structure of myosins. The enthalpy of the thermal unfolding depends on the nucleotides, the conversion from a strongly attached state of myosin to actin to a weakly binding state is accompanied with an increase of the transition temperature which is due to the change of the affinity of nucleotide binding to myosin.

The data support that the strong-binding state and rigor state differ energetically from each other. The differences in the melting temperatures at different intermediates clearly indicate significant alterations in the internal microstructure of myosin head region.

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1-81

FLUORESCENCE CHARACTERIZATION OF THE HUMAN MTH1, AN ANTI-MUTATOR PROTEIN AND ITS INTERACTION WITH NUCLEOTIDE. F. Maraboeuf, H. Yakushiji, Y. Nakabeppu, M. Sekiguchi and M. Takahashi.

Oxidation of dGTP in the cell produces 8-oxo-dGTP. This oxidized nucleotide can be incorporated into DNA during replication, and promotes G:C to T:A transversion mutation which could cause a cancer. Human MTH1 protein selectively hydrolyses 8-oxo-dGTP to 8-oxo-dGMP and thus prevents the mutation. Our aim is to identify the nucleotide binding domain of MTH1 to understand the high specificity of the protein for 8-oxo-dGTP.

We examined eventual involvement of tryptophan residue(s) in the nucleotide binding domain of MTH1 by measuring the tryptophan fluorescence of the protein. dGDP, a non-hydrolizable competitor of 8-oxo-dGTP for the protein was used as a nucleotide.

Addition of dGDP decreases the fluorescence about 40% in intensity and shifts the emission maximum from 345 to 338 nm. To identify the tryptophan residue(s) involved in the binding domain, we further characterized the environment of the residue(s) by dynamical quenching techniques. Anionic quencher I^- decreased the fluorescence around 343 nm while cationic quencher Cs^+ decreased around 350 nm. The results indicate the presence of 3 groups of tryptophan environment. One is well-exposed on the surface of the protein but in negative environment, a second partially buried in positive environment, and a third totally buried. dGDP quenches the fluorescence of the first type.

The nucleotide binding domain is most likely close to the tryptophan residue(s) in negative environment. The analyse of the primary sequence of the protein suggests that Trp 117 and 123 which belong to a cluster of aromatic residues could be in the nucleotide binding domain.

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LIQUID CRYSTALLINE SELF-ASSEMBLIES OF TYPE I COLLAGEN. Martin R, Giraud-Guille M M and Besseau L.

In biological tissues (bone, dermis, tendon, etc...) three dimensional arrangements of fibrillar collagen network follows cholesteric liquid crystalline geometries. To understand how collagen self-assembles *in vivo*, the behaviour of concentrated acido soluble type I collagen solutions (40-80 mg/ml) is studied *in vitro*. The typical cholesteric textures spontaneously obtained are characterized by polarized light microscopy (1). Replicas of platinium shadowed molecules analyzed in transmission electron microscopy show that sonication fractions collagen triple helices, optimizing both in time and space the cholesteric order (2).

In the present work, similar experiences are carried out to study the influence of pepsin digestion on collagen self assemblies. Pepsin treatment removes non-helical terminal peptides of collagen molecules responsible for the formation of covalent crosslinks in fibrils. Observations in electron microscopy revealed that pepsin digestion strongly reduces molecular aggregates in acido soluble solutions. Polarized light microscopy attested that pepsinization optimizes cholesteric order in time and space as sonication does. Furthermore, successive treatments with pepsin and ultrasonic waves on a same collagen solution improved even more the emergence of cholesteric order *in vitro*. Further studies are performed to investigate possibilities of crystalline liquid assemblies with procollagen, the non-processed form of collagen as is secreted by the cell. Contrary to collagen, procollagen has large additional extension peptides which confer on the molecule a high solubility at neutral pH. Microscopic studies of concentrated procollagen in physiological buffer will then provide clues about possible long-range orders established before fibrillogenesis.

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1-83

ENHANCEMENT OF CALCIUM AFFINITY FOR CALMODULIN INDUCED BY TARGET INTERACTIONS. S.R.Martin & P.M.Bayley.

Many peptides, including those derived from the target sequence of skeletal muscle myosin light chain kinase (sk-MLCK), bind strongly to calcium-loaded calmodulin (Ca_4-CaM), with $K_d < 10^{-9}$ M, but bind very weakly to apo-CaM with $K_d > 10^{-3}$ M. The average Ca affinity for CaM alone, $K_{av} = (K_1,K_2,K_3,K_4)^{1/4}$, where K_i are the four stoichiometric Ca binding constants, is ~$2x10^5$ M^{-1}. In the presence of different full length sk-MLCK target peptides, K_{av} is increased by factors of between 20x and 150x, corresponding to a striking Ca induced increase in peptide affinity, in the range of $1.6x10^5$ to $5x10^8$-fold. One of the synthetic model sequences LKLKKLLKLLKKLLKLG binds to Ca_4.CaM with $K_d < 10^{-11}$M, but the enhancement in K_{av} is < 10-fold. Consistent with the above interpretation, we show that this peptide binds to apo-CaM with unusually high affinity ($K_d < 10^{-7}$ M). In the presence of target sequences which bind to both domains of Ca_4.CaM, all four sites bind Ca more tightly; the peptide increases all of the stoichiometric binding constants and reduces the dissociation rates for the Ca ion-pairs in both domains. The intrinsic differences in C- and N-domain affinities mean that intermediate species, such as Ca_2.CaM.peptide can be formed, with Ca binding to the higher affinity C-domain amplified by the peptide, suggesting a possible role in regulation of enzymic activity at intermediate levels of [Ca]. In general, the differences in the Ca affinity of the CaM domains, (plus differences induced by specific mutation of CaM and modification of target sequences), can give rise to multi-phasic Ca saturation curves in the presence of peptide sequences. These curves show how both the [Ca]-range and threshold for Ca regulation of the target enzyme activity can be controlled by sequence variation of residues involved in energetically important calmodulin-target peptide interactions.

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1-84

THE HIV-1 NUCLEOCAPSID PROTEIN, NCp7, IS AN EFFICIENT RNA AGGREGATINGAGENT. S.P. Stoylov, E. Le Cam, D. Coulaud, E. Delain, E. Stoylova, C. Vuilleumier, D. Gérard and Y. Mély

The HIV-1 nucleocapsid protein, NCp7, is a small basic protein characterized by two strongly folded CCHC zinc finger motifs. NCp7 plays an important role in several key steps of the viral life cycle but many of the NCp7 activities, notably the wrapping of genomic RNA and the activation of complementary nucleic acid sequence annealing, are probably linked to a nonspecific binding of NCp7 to its target nucleic acids. The mechanism of these activities is thought to involve an intermediate aggregation of nucleic acids by NCp7. To check and characterize the RNA aggregating properties of NCp7, we investigated by quasielastic light scattering and electron microscopy, the interaction of NCp7 with several model RNAs at various nucleotide to protein ratios, protein concentrations and salt concentrations. Monodisperse and spherical discrete particles with a dense core and a filamentous halo were observed in a large range of conditions. These particles contained a large number of RNA molecules covered by NCp7 molecules and orderly grew with time: the growth kinetics being adequately fitted with a kinetic law used in crystal growth. The particle formation was critically dependent on NCp7 concentration. The particle features also strongly depended on the salt (NaCl and $MgCl_2$) concentrations but depended only moderately on the nucleic acid structure and length. Finally, using various NCp7 derivatives, we found that the basic regions of NCp7, but not the zinc finger motifs were critical in the RNA aggregation. Taken together, our data indicate that NCp7 is an efficient nucleic acid aggregating agent and strengthen the hypothesis that aggegation may be involved in some NCp7 functions.

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1-85

CONTROL OF CELLULAR MOTILITY THROUGH THE CONFORMATIONAL CHANGES OF CHEY. F.Mérola, T. Tolstykh, M.Levit, K.Martinez, D.Deville-Bonne, and J.B.Stock

Chemotaxis in *E.Coli* functions through the reversible phosphorylation of a small response regulator called CheY. Phosphorylation at Asp57, in the presence of Mg^{2+}, induces a conformational change that allows the protein to bind to components of the flagellar motor, and control motility. We have performed time-resolved polarized fluorescence experiments of the single Trp58 residue, located adjacent to the active site, to characterize the conformational transitions of CheY.

The fluorescence decays of the free protein reflect a high degree of local structural micro-heterogeneities, together with a large rotational mobility of the Trp side chain. The angular freedom of rotation of the Trp58 emission moment, estimated from the (sub)nanosecond intermediate components of the anisotropy decays, approaches that observed for some molten globule proteins. Aside from unspecific dynamic quenching, the detailed structural and dynamic properties around Trp58 are not perturbed by the binding of Mg^{2+} at the nearby active site. On the contrary, phosphorylation of the CheY+Mg^{2+} complex induces a large population (30%) of a new highly quenched species. Removal of Mg^{2+} by addition of EDTA suppresses this quenched population, while 20% of a new long fluorescence lifetime appears. This species would then correspond to a conformation of phosphorylated CheY in which Trp58 approaches Mg^{2+} within its active volume of quenching. Further studies include now mutants unable to undergo either the phosphorylation step (D57N) or the consecutive functional conformational switch (K109R).

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1-86

SYNTHESIS AND CONFORMATIONAL STUDY BY CIRCULAR DICRHOISM OF A 30-MER PEPTIDE CONTAINING B- AND T-CELL EPITOPES OF HEPATITIS A AND B VIRUSES

M. José Gómara*,**, Concepción Mestres**, Victoria Girona**, M.Asunción Alsina** and Isabel Haro*

The synthesis of the pentadecapeptide (2F10 peptide: AVYYCTRGYHGSSLY) reported by Rajadhyaksha et al (1) capable of mimicking the group-specific "a" determinant of human hepatitis B surface antigen at both the B- and the T-cell level was carried out. In order to potentiate the immunological properties of the hepatitis A virus related dodecapeptide (VP3(110-121): FWRGDLVFDFQV) previously synthesized in our laboratory (2) a 30-mer peptide sequence having both 2F10 and VP3(110-121) peptides in two different orientations (BT and TB) is described. Moreover, a conformational characterization by circular dichroism has been performed. The quantitative analysis of experimental data has been carried out using Lincomb, Contin and K2D computer programs.

(1) M. Rajadhyaksha et al, Proc. Natl. Acad. Sci., USA, 92, 1575 (1995)
(2) J.A. Pérez et al, Biomedical Peptides, Proteins & Nucleic Acids, 1, 93 (1995)

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1-87

THE DECAPSIDATION OF A SMALL ISOMETRIC PLANT VIRUS, TURNIP YELLOW MOSAIC VIRUS : A DIFFERENTIAL MICROCALORIMETRIC STUDY. B. MICHELS* and J. WITZ**

Previous studies have shown that the heat-induced decapsidation of TYMV occurs in three successive irreversible steps : 1) release of the RNA through a hole in the capsid, leaving behind an empty shell (ATC-T); 2) dissociation of these empty shells; 3) denaturation of the dissociated components. An exothermic process accompanies the first step, which probably corresponds to an expansion of the capsid preceding decapsidation (Mutombo et al., 1993, *Biochimie* **75**, 667-674).

We have investigated the thermostability of natural empty shells (NTC) and artificial empty shells (ATC) obtained by various treatments of purified virions. NTC and ATC-A obtained by alkaline treatment exhibit only one transition at a temperature corresponding to step 3). ATC-F, obtained by freezing and thawing, exhibits transitions 2) and 3). Unexpectedly, once purified, ATC-T behaves as NTC, providing evidence that empty shells may undergo a slow conformational change after release of the RNA. This difference in thermostability does not correlate with the presence or absence of a hole in the protein shell, since the presence of such a hole is documented for ATC-A as well as for ATC-F.

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1-88

STUDIES OF THE CONFORMATIONAL STABILITY OF APO-NEOCARZINOSTATIN. E. Adjadj, E. Quiniou and J. Mispelter.

Apo-neocarzinostatin is a small protein of 113 residues which transports and protects an antitumoral antibiotic agent. The structure of this protein is based on a 7 stranded β barrel similar to the V_H immunoglobulin folding pattern.

The conformational stability of the protein, during thermal unfolding, has been observed by circular dichroïsm, fluorescence and NMR. The temperature dependence of the proton NMR spectra reveals that denaturation is initiated at a particular polypeptide region close to a critical disulfide bridge in the protein. The reduction of this bond leads indeed to a dramatic decrease in the structural stability of the protein. More generally, the importance of the residues involved in the coherence of the β barrel moiety have been determined from the analysis of the segmental motions given by ^{13}C NMR. In particular, relaxation data obtained for some methyl carbon nuclei allows one to distinguish the side chains implicated in hydrophobic interactions and those implicated in the ligand binding site.

These data will be of importance for the design of mutants or similar polypeptide chains, based on the apo-NCS core, with the object of constructing efficient transporting proteins for therapeutic agents or as models, or replacement, of specific immunoglobulin domains.

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1-89

THE PROPERTIES OF PRION PEPTIDE AMYLOID IN SOLUTION P.K. Nandi.

The prion desease based on 'protein only' hypothesis involves a three step process where the normal cellular prion, PrP^{C}, rich in α-helices, converts to β-sheet rich scrapie prion, PrP^{SC}. *In vivo* proteolysis of PrP^{SC} remove the N-Terminal peptide till 89th amino-acid which results in the prion amyloid PrP27-30 with further enrichment of β-sheet and is considered to be involved in the neurodegeneration of human and certains animals including sheep, goat etc. The prion peptide 106-126 forms amyloid which is toxic to primary brain cells in culture and has been used as a model to study prion 'infection'. We have studied the solution properties of the peptide amyloid by fluorescence and turibidity measurements and compared them with the available data for prion amyloid 27-30. The formation of the peptide amyloid accompanies the accessibility of hydrophobic groups as in PrP27-30 amyloid. The peptide amyloid is most stable arround pH 5, dissociates in lyotropic salts and guanidine HCl (G) solutions and stabilised by cosmotropic ions. Dissociation of the peptide amyloid in G mimicks the behaviour of the prion PrP27-30 amyloid. The amyloid formation involves both hydrophobic and polar regions of the molecule probably by a shift in the relative positions of the molecular surfaces to overcome electrostatic repulsion of the positively charged PrP106-126. Dissociation of the peptide amyloid in electrolyte solution probably involves both water structure effect and ion-amide dipole interaction.

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1-90

THE STRUCTURE OF CARBOHYDRATE CHAINS OF α_1-ACID GLYCOPROTEIN. SURFACE-ENHANCED RAMAN SCATTERING SPECTROSCOPY STUDY. V. Oleinikov, A. Feofanov, A. Ianoul, E. Kryukov, S. Shiyan, N. Bovin, I. Nabiev.

As was established earlier, the property of α_1-acid glycoprotein (AGP) to inhibit the lymphocyte proliferation is attributed to its carbohydrate chains which are presented by complex type oligosaccharides (di-, tri- and tetraantennary chains). The goal of this study was to define the role of structure and antennary of AGP carbohydrate chains in the processes of polyvalent interaction with the cell surface. Two experimental approaches were used: (1) translocating AGP carbohydrate chains from peptide core onto soluble polyacrylamide and creating "pseudo-AGP" neoglycoconjugates with variable density of oligosaccharides, and (2) separating glycoforms of AGP with different content of di-, tri, or tetraantennary carbohydrate chains. The structural features of carbohydrate chains of neoglycoconjugates and AGP glycoforms (conformational flexibility, location and orientation of some carbohydrate terminal groups, which can be either accessible to interact or screened by other molecular fragments) were determined by surface-enhanced Raman scattering (SERS) spectroscopy. As we have shown, SERS spectroscopy provides an unique possibility to detect selectively and sensitively (up to 10^{-5} M concentration) the signal of sialic acids (SA), which are the terminal residues of glycoproteins, and thereby to define the accessibility of SA to interact. Spectroscopic data were compared with the results of biological studies of AGP, its glycoforms and synthetic conjugates. To estimate immunological activity of the compounds the proliferation response of peripheral human blood lymphocytes on the phytohemagglutinin was used. The dominance of diantennary T-type conformation of AGP carbohydrate chains in interaction with the cells as well as with SERS-active surface was shown.

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1-91

X-RAY STRUCTURE OF A METASTABLE MYOGLOBIN FE(II)-LS INTERMEDIATE. A. Ostermann, D.C. Lamb, V.E. Prusakov and F. Parak.

A metastable Fe(II)-ls myoglobin state can be formed by reduction of Fe(III) met myoglobin with X-rays at 77 K. Above 150 K, this nonequilibrium state of myoglobin begins to relax to the deoxy equilibrium conformation. This relaxation is an example of a r→t transition. The relaxation of the Fe(II)-ls intermediate is nonexponential in time as recently shown by Mössbauer[1]- and optical spectroscopy. The dynamics of protein relaxation provides insight into how a protein functions. The X-ray structure of the intermediate myoglobin state has been determined at 115 K to support the spectroscopic data and their interpretation. After irradiating a ^{57}Fe(III) met myoglobin crystal at 90 K for 90 hours, the Mössbauer spectrum was measured to verify that the Fe(II)-ls state was created. After data collection for the structure determination this control measurement was repeated. 93 % of the possible reflections have been collected to a resolution of 1.4 Å with an overall redundancy of 4.0. As a reference the structure of Fe(III) met myoglobin was determined to the same resolution using an unirradiated crystal from the same crystallisation batch. The crystal structure of the metastable Fe(II)-ls state shows that the water molecule which is present in the met myoglobin form is still bound to the heme iron in the Fe(II)-ls form and the globin structure remains practically in the "frozen" initial met-myoglobin conformation. [1] Prusakov VE, Steyer J, Parak FG (1995) Mössbauer Spectroscopy on Nonequilibrium States of Myoglobin: A Study of r-t Relaxation. Biophys. J. 68:2524-2530

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1-92

GLYCOSYLATION AND LOCALIZATION OF THE RECOMBINANT EXTRACELLULAR DOMAIN OF THE PORCINE LUTROPIN RECEPTOR EXPRESSED IN BACULOVIRUS-INFECTED INSECT CELLS. E. Pajot-Augy, V. Bozon, J.J. Remy, L. Couture and R. Salesse.

Overexpression of the porcine lutropin receptor (pLHR-297) ectodomain has been achieved using the baculovirus-insect cells system, but mostly in an aggregated, non-secreted, inactive form, with an apparent M_W of 48kDa on SDS-PAGE gels. Another baculovirus, with the same cDNA under the control of the earlier and weaker P10 promoter instead of that of polyhedrin, enabled a moderate level of secretion of recombinant ectodomain in a biologically active form, with an apparent M_W of 58kDa. The glycosylation of pLHR-297, electroeluted after electrophoresis separation, was studied by means of endoglycosidase treatments and biotinylated-lectin blotting analysis. Deglycosylation was followed after SDS-PAGE by means of silver staining or Western blotting with a monoclonal antibody detecting the denatured receptor ectodomain. pLHR-297 glycosylation seems to be limited to the high-mannose type, specifically and entirely removed by endoglycosidase H, and hardly displaced from Concanavalin A binding by tremendous amounts of the competing carbohydrate. However, all 6 lectins used displayed a specific binding to pLHR-297, which also indicates an heterogeneous glycosylation of the recombinant receptor. The recombinant receptors within the infected Sf9 cells are being visualized by immunofluorescence, immunogold staining, and gold-labeled lectin binding, to compare their localization within the secretory pathway, and to characterize the glycosylation of pLHR-P10-297 as compared to pLHR-297.

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1-93

MÖSSBAUER STUDY ON PHOTOSYSTEM II MEMBRANE FRAGMENTS (PS II MF) A. Garbers, J. Kurreck, F. Reifarth, O. A. Iakovleva, G. Renger and F. Parak

Samples of PS II MF from spinach grown hydroponically on ^{57}Fe have been investigated by Mössbauer spectroscopy at several temperatures. The Mössbauer absorption area ratio for the cytochrome (cyt) b_{559}- and the nh-iron subspectra indicates two cyt irons per one nh iron. The cyt-Fe shows two species which differ in their oxidation states, i.e. Fe(II) S=0 and Fe(III) S=1/2, which reflects the presence of a high and low potential form. Time-resolved flash-induced fluorescence spectroscopy of PS II MF showed a specific temperature dependence of electron transfer from plastoquinone Q_A^- to Q_B in the interval 230-270 K [1]. A comparison with mean square displacements, $\langle x^2 \rangle$, obtained from the Lamb-Mössbauer factor yielded a strong correlation of the photoinduced electron transfer to the onset of protein specific motions. The investigation of the oxidized cyt-Fe reveals a lower slope of the linear part of the mean square displacement in comparison with the Fe(II) species. Mössbauer spectroscopy proved that the isolation of the D1/D2/cyt b_{559}-complex depletes the reaction center of all detectable nh-Fe. All cyt b_{559} are found in the low potential form with the iron centre in the Fe(III) low spin state. The magnetically splitted spectra of ferric cyt were analyzed using the model of Griffith taking into account spin-lattice relaxation. [1] Renger, G.; Haag, E.; Gleiter, H.; Reifarth, F. (1993) Z. Naturforschung 48c, 234-240

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1-94

2D-NMR STUDIES OF THE 141-159 FRAGMENT PEPTIDE OF VP1 COAT PROTEIN FROM FOOT-AND-MOUTH DISEASE AND ITS RETRO-INVERSO ANALOGUE. MC PETIT, A. PHAN CHAN DU, P. ORLEWSKI, N. BENKIRANE, G. GUICHARD, JP BRIAND, S. MULLER and MT CUNG

A major problem limiting the use of peptides as vaccines is their instability. Most endogenous and exogenous biologically active peptides are short-lived molecules that are rapidly degraded *in vivo* by proteases. However, this instability can be overcome by using mimetics such as retro-inverso peptides. It has been showed that retro-inverso peptidomimetics of the major antigenic site of foot-and-mouth disease virus presents an enhanced immunogenicity and cross-reactivity with antibodies raised against the parent L peptide (1).

Structural studies in aqueous solution of the retro-inverso peptide and its parent peptide were carried out by NMR spectroscopy and molecular modeling. Restraints found when studying the parent L peptide were mostly short range (i, i+1) and (i, i+2) NOEs. Only two long range NOEs were found between residues Arg145 and Val155, reflecting the low structure level of that peptide. In the study of the retro-inverso peptide, numerous (i, i+2), (i, i+3) and (i, i+4) NOEs together with long range NOEs were observed, highlighting the better structuration of the analogous peptide, which might be an explanation for the enhancement of immunogenicity of that peptide.

Reference

1. S. Muller, et al., *Peptide Res.* **8**, 138-144 (1995).

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1-95

IN13 : A BACKSCATTERING SPECTROMETER AT THE ILL FOR STUDYING THE FUNCTIONAL DYNAMICS OF PROTEINS. Ursula Lehnert, Claude Pfister, Anton Heidemann and Giuseppe. Zaccai.

Incoherent neutron scattering in proteins concerns essentially H-atoms, uniformly spread in molecules. In the nano-picosecond time range, their movements reflect those of the residues to which they are linked. Experiments on bacteriorhodopsin, the light-driven proton pump in *Halobacterium salinarium*, were the first to correlate the appearance of anharmonic movements of high amplitudes in the protein with biological function (Ferrand et al, PNAS 90, 9668-9672,1993). D-atoms scatter neutrons about 40 limes less, and pecific H/D labeling allows to selectively observe the H-labeled part of a protein. The feasability of protein preparation and of analysis by elastic incoherent neutron scattering has been shown on bacteriorhodopsin (Réat, Thesis, Grenoble1997). The analysis of atomic movements in bacteriorhodopsin reveals a "hard core" (the retinal pocket and the extracellular half of the transmembrane helices) in a "softer body". This has been related to the vectoriality of proton transfer in the protein. It is of almost interest to carry out similar studies in proteins with different functions (ion transport, enzymatic catalysis, electron transfer) and compare the requirements for "soft" or "hard" movements.

The backscattering spectrometer IN13 at the ILL appears as the most suited for such studies, because it allies a Q-range of up to 5Å^{-1} (corresponding to observable movements of about 1Å) and an energy resolution of about 10μeV (corresponding to observable caracteristic times of 0.4 ns). It is the only existing instrument with such characteristics, and complements the other spectrometers with different Q-ranges and energy resolutions. It has been shown that each set of characterisitics allows the description of a different subpopulation of movements in proteins (Réat et al, J. Biomol. Struct. Dyn.. 1997).

IN13 has been made functional again, in the frame of a project for a "Collaborating Research Group". Under the conditions of normal neutron flux, acquisition of elastic scattering intensities as a function of Q (32 values in the à.6-5 Å^{-1} range) and of temperature (60 values in the 20-320K range) on a biological sample containing about 150 mg protein with natural H-abundance necessitates 1-2 days.

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1-96

COMPARATIVE TIME-RESOLVED FLUORESCENCE STUDY OF FREE OR LIPOSOME-BOUND ANNEXIN V. C. Pigault, E. Piémont, J.M. Freyssinet, D. Gérard and A. Follenius-Wund.

Annexin V belongs to a widely distributed family of proteins which interact with acidic phospholipids in a Ca^{2+}-dependent manner. Previous crystallographic and steady-state fluorescence studies have shown that its unique Trp-187 residue lies in the hydrophobic core of the protein in the absence of Ca^{2+}. This residue becomes more exposed to the solvent when annexin V binds to acidic liposomes in the presence of Ca^{2+}. In the present study, the involvement of Trp-187 in the binding of annexin V to liposomes was investigated by time-resolved fluorescence spectroscopy.

Annexin V was purified from human placenta. The large unilamellar phospholipid vesicles were prepared by extrusion. Fluorescence lifetime measurements were performed with a pulsed frequency-tripled titane-sapphire laser, with the excitation wavelength set at 295 nm.

The fluorescence decay of Trp-187 of free annexin V could be described by the sum of three exponential components, each corresponding to one class of Trp residues. The major two classes were embedded in the protein matrix since their maximum emission wavelengths (λ_{max}) were at 325 and 320 nm respectively. The same characteristics were obtained for annexin V in the presence of pure phosphatidylcholine liposomes, confirming that there is no binding with neutral phospholipids. The fluorescence decay of annexin V bound to acidic liposomes was adequately fitted with only two components. The decay-associated spectra revealed the existence of an equilibrium between two classes of Trp residues : one class corresponds to Trp residues located in a partially solvent-exposed position (λ_{max} = 340 nm), the other to a buried position (λ_{max} = 325 nm).

These results show that, in peripheral annexin membrane interactions, some Trp-187 residues remain at the interface whereas the other penetrate into the phospholipid bilayer.

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1-97

THE INTERACTION OF CALMODULIN WITH FLUORESCENT LABELLED TARGET PEPTIDES. S.K.Pirzaman, G.Marriott* and P.M.Bayley

Calmodulin (CaM) is an intracellular calcium-binding protein responsible for the regulation of a variety of structurally and functionally diverse enzymes. In this study, two cysteine-containing variants of the 18-residue peptide (WFF) representing the CaM binding sequence of skeletal muscle myosin light chain kinase (peptides WFF-C0 : GCKRRWKKNFIAVSAANRFK and WFF-C8 : KRRWKKNCIAVSAANRFK) have been synthesized to explore the use of fluorescent labelling in CaM-target peptide binding studies. The cysteine-reactive probe used is dansyl maleimide (DM), whose fluorescent properties are highly dependent on its environment. The dissociation constants of these peptides for 1:1 complex formation with Ca_4.CaM, prior to labelling, are (like WFF) in the pM range, but the affinity decreases by a factor of ~1000 upon covalent attachment of DM. The fluorescent intensity of the labelled peptides upon binding to CaM is enhanced 20x in the case of WFF-C0-dansyl (λ_{max} 489nm), and 8x in the case of WFF-C8-DM (λ_{max} 511nm). This suggests that the N-terminus of the peptide in WFF-C0-DM, is more buried in comparison with WFF-C8-DM which may be interacting with the more exposed central helix of calmodulin. Fluorescence resonance energy transfer (FRET) measurements show 60% energy transfer from tryptophan to dansyl in WFF-C0-DM and 70% in WFF-C8-DM when CaM binds in a 1:1 complex, corresponding to donor-acceptor distances of 21 A° and 19 A° respectively, based on κ^2= 2/3 and measured R_0= 22 A°. The higher FRET from peptide WFF-C8-DM with a T26W (VU-1) CaM mutant (compared with WFF-C0-DM) is consistent with the peptides adopting the expected orientation with respect to the two domains of calmodulin.

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1-98

CHOLESTEROL - ACYL PROTEIN INTERACTIONS. A ^{2}H-NMR STUDY OF THE ACYL GRAMICIDIN A/ DMPC/ CHOLESTEROL SYSTEM. T. Pott, T.C.B. Vogt, J.A. Killian and B. de Kruijff.

Quite a large number of membrane proteins are coupled covalently to an acyl chain. In the case of palmitoylated proteins, the acyl chain moiety is usually located next to the transmembrane segment of the protein. But despite the natural abundance of acylated membrane proteins, little is known about function and behavior of the membrane inserted acyl chain. Herein we studied the influence of cholesterol on the palmitoyl-gramicidin A (pGA)/ dimyristoylphosphatidyl-choline (DMPC) system. Gramicidin A (GA), a hydrophobic transmembrane helical peptide, is amongst one of the best studied peptides in respect to protein-membrane interaction. An acyl chain can easily be attached to the terminal ethanolamine of GA and pGA was considered to provide a good model for acyl proteins. ^{2}H solid state NMR on a deuterium labeled acyl chain is a powerful technique to study the dynamical behavior of a membrane inserted acyl chain. It was found that the covalently attached acyl chain of pGA senses less the presence of cholesterol than free fatty acid or the fatty acyl chains of the phospholipid. This result is rather puzzling in regard to the proposed preferred interaction of cholesterol with GA. It is suggested that the dynamics of the pGA acyl chain are dominated by the proximity of the GA helix rather than by the membrane compound cholesterol.

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1-99

POPULATIONS OF HYDROPHOBIC AMINO ACIDS IN GLOBULAR DOMAINS OF PROTEINS. A.Poupon & J.P. Mornon.

We have performed a study of the distribution of hydrophobic amino acids within protein cores of known structure in order to extend an observation made a few years ago. Indeed, the comparison of sequences from pairs of structurally similar domains by the 2D Hydrophobic Cluster Analysis (HCA) method, led to distinguish two populations of hydrophobic amino acids. The first one has a low accessibility for the solvent, the second one a significantly higher accessibility. Two main regions of protein globular domain cores are consequently distinguished from sequences only.

In order to confirm this property of globular domains we enlarged the study to non redundant families of similar domains instead of pairs. The previous observation was widely confirmed. The study of families with more than 10 members shows that the two populations of hydrophobic amino acids could be separated through the comparison of only a few members of each family. We show that, if the firstly compared sequences are well chosen, the addition of new sequences to the family brings no or few more information.

This study shows that the small number of conserved hydrophobic positions (positions only occupied by hydrophobic amino acids) constitutes a signature of the subtle structural differences between the proteins of the considered family. This criterion is independent from the sequence identity rates, and from the r.m.s. between the corresponding structures. Such a criterion, determined from the sequences only, gives an estimation of the "distance" between subfamilies of proteins.

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1-100

(TRP/TYR)-PRO MOTIF - POTENTIAL NUCLEATION SITE FOR THE TURN CONFORMATION IN PROTEIN FOLDING. J.Poznański

NMR studies on the three groups of peptides: Trp/Tyr-(Pro)$_n$-Tyr/Met (WP_nY, WP_nM, YP_nM) in aqueous solution demonstrated that *cis-trans* equilibrium on the X_1-Pro_2 peptide bond is abnormally shifted towards the *cis* isomer (up to 75%). Moreover, conformational flexibility of the N-terminal sidechain is significantly restricted with a dominant population of the χ_1(*trans*) rotamer. Analysis of the pH dependance and influence the **X** sidechain size strongly suggest hydrophobic mechanism of stabilization of the N-terminal fragment in $X_1(\chi_1(trans))$ $Pro_2(\omega(cis))$ conformation. Molecular Dynamics simulations performed for WPY peptide in the presence of range distance constraints deduced from 200ms ROESY spectra lead to stable structure resembles part of the β turn VI motif in protein fold accompanied by the close contacts of tryptophan sidechain and proline pyrrolidine ring. Proton chemical shift calculations performed for this structure are in a good agreement with the unusual values measured experimentally. Calculations of the solvation free energy based on the solvent accessible areas confirm the hydrophobic character of observed phenomena. Such type of stabilization has been reported for longer linear peptides exhibiting structures of type VI turn. Postulated X_{i-1}-Pro_i structure is also overrepresented in the known protein 3D structures. In the Protein Data Bank among the 73 found WP(ω(*cis*)) fragments 85% exhibit close contact of imidazole and pyrrolidine rings. The ring-ring interaction is supposed to stabilize also other types of protein loops; e.g.Tyr_{17} Pro_{25} interaction keeps ends of the large solvent exposed loop in maize nonspecific Lipid Transfer Protein. Observed phenomena may give new view to the understanding mechanism of protein folding, prooving that even in early states of protein folding significant populations of the native structured turns exists.

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1-101

NATURAL ABUNDANCE ^{13}C NMR OF A NON SPECIFIC TRANSFER PROTEIN (NS-LTP) J. Mispelter(1), P. Sodano (2), A. Caille (2), D. Marion (3) and M. Ptak(2)

Wheat ns-LTP is a small protein of 90 residues which bind various hydrophobic molecules and phospholipids. Transfer of lipids has been proved *in vitro* but the *in vivo* function of the protein remains questionable. The protein is highly soluble and very stable in solution allowing the record of ^{13}C NMR data at the natural abundance level. Their chemical shifts are relevant to structural properties while relaxation data provide unique local insight into the internal motion on the ps to ns time scale.

Most of the resonances of the protonated ^{13}C (Cα and side chains except aromatics) have been assigned using three experiments: hsqc, hsqctocsy and a double dept experiment with 90° transfer pulses. The backbone ^{13}C chemical shifts exhibited deviations from random coil which are related to the secondary structure, as expected.

Internal motions for the backbone nuclei, has been investigated from the heteronuclear ^{13}C-{^{1}H} data. These preliminary data provide a qualitative index for the ps to ns local motions. As a result, the C terminal residue exhibit less motion than the N-terminal one. The most restricted region involves residues 12 to 38 and 64 to 70. An increased motion is observed for residues 50 to 63 and for the 6 C terminal residues. In addition, specific high motional properties are found for residues 49 and 60. These observations do not correlate specifically with the secondary structure of the protein, nor with the rmsd among the NMR structures. These experiments provide a first insight into the backbone dynamic of this protein and the assignment of side chain carbons open the possibility for investigating the internal motion at these sites.

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1-102

DETERMINATION OF INTERSPIN DISTANCES BETWEEN BOUND SPIN LABELS TO STUDY PROTEIN STRUCTURES AND STRUCTURAL CHANGES. N. Radzwill, W. Thevis, V. Lenz, D. Brandenburg, F. Anston, G. Dodson, A. Wollmer , K. Gerwert and H.-J. Steinhoff

Membrane proteins present a difficult problem for structure determination. With a few exceptions, X-ray diffraction data are not available due to unsolved problems of crystallisation. Two-dimensional NMR techniques are not applicable due to the lack of high resolution spectra. The study of interspin distances in proteins with two or more nitroxide spin labels attached to specific sites is an alternative approach to obtain structural information on proteins in solution or membrane proteins. This method has been applied to spin labeled insulins in the R6 and T6 states and to different intermediates of spin labeled bacteriorhodopsin (BR). The EPR line broadening due to dipolar interaction was determined by the fitting of simulated EPR powder spectra to experimental spectra, which were measured at temperatures below 200 K to freeze the protein motion. Resulting interspin distances determined for cristallized insulins in the R6 and T6 structure were in excellent agreement with the structural data obtained by X-ray cristallography and modelling of the spin labeled samples. The EPR experiments revealed slight differences between crystal and solution structures of the B-chain N-termini in hexameric insulins in the R6 and T6 states and additionally provided information about their flexibility. Distance changes between the cytoplasmic loops CD and EF were detected after freezing of the BR in different intermediates. Characterization of the frozen intermediates by FT-Raman spectroscopy revealed that the conformational change of the labeled loops occurs during the M to N transition.

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1-103

NUCLEOTIDE BINDING SITE IN NATIVE AND IN W227Y CREATINE KINASE MUTANT PROBED BY PHOTOCHEMICAL RELEASE OF NUCLEOTIDES AND FTIR SPECTROSCOPY. C. Raimbault, C. Perraut, O. Marcillat, R. Buchet and C. Vial.

FTIR spectroscopy was used to monitor distinct structural changes induced by the binding of photochemical released ligands (ATP, ADP and Pi) to native creatine kinase and to a W227Y mutant. The changes observed in the 1600-1670 cm^{-1} region of our reaction-induced difference infrared spectrum, were assigned to carbonyl groups of peptide backbone involved in the binding of ligands. In addition, the 1560-1590 cm^{-1} region was affected, indicating that carboxylate groups of Asp or Glu are also implicated in the binding of ligands. These infrared changes decreased strongly in the case of W227Y mutant, indicating that nucleotide binding site is impaired. The magnitude of the decrease was not the same for every band. In particular, the decrease in magnitude of the 1625-1627 cm^{-1} band was strongest, suggesting that the mutation affected differently molecular interactions involving carbonyl and carboxylate groups.

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1-104

THE PROTON RELEASE MECHANISM OF BACTERIORHODOPSIN MONITORED BY TIME-RESOLVED STEP-SCAN FT-IR SPECTROSCOPY

Robin Rammelsberg, Gregor Huhn, Mathias Lübben, and Klaus Gerwert

The light-induced proton pump mechanism of the membran protein bacteriorhodopsin is investigated with nanosecond time-resolved step-scan FT-IR spectroscopy (1,2).

During bacteriorhodopsins` photocycle an unknown group XH releases a proton into the extracellular medium (3). To identify this group the residues arg82 and glu204, which are discussed as XH, are replaced by side-directed mutagenesis. These mutations affect the time of the proton release as measured with pH-sensitive dyes. Nevertheless the time-resolved FT-IR measurements analysed by a global-fit do not show time-courses, which can be assigned to a transient deprotonation of glu204 or arg82 during the photocycle. Therefore our results support the model of a delocalized proton release group consisting of a hydrogen-bonded network, which is formed by several residues and water molecules (4,5). The transient deprotonation of this network can be followed by the continuum absorbance above 1800 cm^{-1}.

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1-105

THERMAL STABILITY OF THE PROTEIN HMG1: A CALORIMETRIC STUDY. D. Locker, M. Leng, J. Ramstein

HMG1 is an abundant non-histone like chromosome protein which binds selectively on distorted regions of double-stranted DNA molecules. HMG1 presents three domains, the so-called domain A and B and an acidic C terminal tail. Domain A and B have a strong sequence homology and the structures of these two proteins in their isolated state have been elucidated by 2D NMR ; both proteins present a structure with three alpha-helices. Using differential microcalorimetry with temperature scanning (DSC) and circular dichroism we have studied the thermal stability of domain A and B and of the protein A-B where the two domains are linked by 7 residues long peptide chain. The thermal unfolding data for domain A and B are in agreement with two state melting processes characterized by the same melting temperature (Tm=41°C) and by nearly equal denaturation enthalpies (Δ Hden.=50 Kcal/mol.). On the other hand the thermal denaturation of the protein A-B indicates the presence of two independent folding units whose stability and denaturation enthalpies are comparable to the corresponding values obtained for the isolated domain A and B. Therefore we propose to identify the two independent cooperative units as domain A and B. This means that both domains maintain their structure in the protein A-B.

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1-106

CORRELATION BETWEEN FLUORESCENCE PARAMETERS AND STRUCTURAL CHARACTERISTICS OF MICROENVIRONMENT OF TRYPTOPHAN RESIDUES IN PROTEINS. E.A. Burstein and Ya.K. Reshetnyak

In order to test and develop the hypothesis of discrete structural, physical and spectrofluorescent states of tryptophan in proteins [1] it was carried out cluster analysis of emission and structure parameters of a great number of tryptophan residues in proteins. For this purpose, tryptophan fluorescence spectra of ca.140 proteins were resolved into log-normal components [2, 3] and assigned to individual tryptophan residues. The histogram of occurrence in proteins of tryptophans with various emission maximum positions (from 308 to >350 nm) clearly confirmed the hypothesis of discrete states. The correlations were revealed between spectral maximum position and such features of microenvironment of individual indolic fluorophores as accessibility to solvent of tryptophan residue of some indolic atoms, environment density and B-factors etc. We analyzed also the presence of eventual partners in excited state H-bonding of individual indolic atoms.

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A TIME-DOMAIN EPR STUDY OF THE STRUCTURAL CHANGES OF BACTERIORHODOPSIN DURING THE PHOTOCYCLE. T. Rink, J. Riesle, D. Oesterhelt, K. Gerwert and H.-J. Steinhoff

It is widely accepted that bacteriorhodopsin (BR) undergoes a major conformational change during the second half of its photocycle. Various diffraction experiments show that these changes occur near helices B, C, F and include a prominent reordering of helix G. While these techniques reveal valuable information about the location of the structural changes, they fail in giving detailed kinetic data. Very recently, however, it has become feasible to resolve the conformational dynamics of BR in space and time simultaneously by means of time domain electron paramagnetic resonance (EPR) spectroscopy of site-directed spinlabeled BR mutants. Due to the anisotropy of the hyperfine splitting, the spectrum of the protein bound nitroxide spinlabel depends on its mobility and is therefore sensitive to the local protein conformation. We used cysteine mutants of BR in positions 36, 38 (cytoplasmic AB-loop), 46 (proton uptake channel region of helix B) and 161 (cytoplasmic EF-loop) to attach a MTS spinlabel to the protein. A transient change in EPR absorption following flash photolysis reveals a reversible structural change of the protein near position 36, 46 and 161. In mutant D38C, no conformational changes occur and consequently no transient EPR signal change has been detected. In order to assign the structural changes to any of the known photocycle intermediates, we measured the absorption kinetics of each mutant in the visible and applied a global fitting procedure to both VIS and EPR data. Our results show that the structural change of the AB-loop and channel region starts during the M to N transition, whereas the change detected at position 161 is already present in M. The transient EPR signal decays in all cases with the recovery of the BR ground state.

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1-108

CONFORMATIONAL CHANGES OF THE *E. COLI* CHAPERONINE GROEL AND GROES DURING THE REFOLDING PATHWAY OF SUBSTATE PROTEIN INVESTIGATED BY SMALL ANGLE NEUTRON SCATTERING. M. Rößle, R. Stegmann, E. Manakova & H. Heumann.

Molecular chaperonines like the GroEL and GroES are proteins which bind incorrectly folded polypetides, such as denatured proteins or proteins in *statu nacendi*, preventing aggregation and then mediates their refolding in an ATP-dependent process. During this refolding reaction the co-chaperonin GroES binds to the GroEL and form a stabil complex. We used small angle neutron scattering to to get information about the conformational changes and the spatial arrangement of both components upon complex formation by D-isotopic labeling. This method permits the seperate observation of both proteins in the complex without crystallisation. We investigated differences between the proteins isolated and in the complex indicating conformational changes during the binding reaction of GroEL to GroES. In order to interpretate the observed differences, the crystall data of isolated GroEL and GroES was used to calculate theoretical scattering data. By comparison the scattering data and the data of the theoretical calculations we were able to derive models for the GroEL and the GroES both *in situ*. The center-to-center distance of GroEL and GroES was determined using the „Stuhrmann"-approach. From these data, we were able to make suggestions for the structure of the GroEL/GroES complex.

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1-109

FURTHER CHARACTERIZATION OF THE ACTIN-SMOOTH MUSCLE CALPONIN INTERFACE. A. Bartegi, C. Roustan, Y. Benyamin, R. Kassab and A. Fattoum.

Calponin (CaP) is an actin-,calmodulin-, and tropomyosin-binding 34kDa protein present in smooth muscle and nonmuscle cells. CaP inhibits, *in vitro*, the actin-activated ATPase activity of myosin. Both binding to actin and inhibition of the ATPase can be relieved upon the interaction of CaP with calcium-binding proteins as well as by its specific phosphorylation. Thus, CaP has been proposed as a thin filament based regulatory component in the contractile machinery. We previously reported, using a zero-length cross-linker that the C-terminal region of actin spanning residues 326-355 in the subdomain-1, is involved in the interaction with CaP. This region of actin was proposed as being an important part of the strong myosin binding site. More recently, using proteolytic cleavage, synthetic peptides and recombinant fragments, we have characterized the CaP polypeptide A145-I163 as the minimum sequence necessary for actin binding and acto-S1 inhibition. In this study, we have used polarization fluorescence, co-sedimentation, crosslinking reactions and quenching analysis to provide information regarding the conformational changes in chicken gizzard CaP induced by salts, as well as, by its interaction with actin. It is concluded that the binding of F-actin to CaP changes the environment of the fluorophore linked to the Cys 273 in the C-terminal part of CaP leading to a significant decrease of the acrylamide-induced quenching. The labeling of Cys 273 reduces the affinity of CaP for actin and the stoichiometry of the binding was found to be dependent on salt concentration. Crosslinking studies and fluorescence energy transfer measurements are underway to determine more precisely the properties of the interaction of actin with the C-terminal domain of CaP.

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1-110

STRUCTURAL DYNAMICS OF PROTEINS AS DETERMINED BY MULTIDIMENSIONAL NMR SPECTROSCOPY.

H. Rüterjans

Refined solution structures of various proteins were obtained using NOE distances and homo- and heteronuclear ^3J couplings from ^{13}C and ^{15}N enriched protein samples. It was possible to describe conformational equilibria within the investigated proteins. Some of these conformations were not observed in corresponding crystal structures.

From a determination of relaxation times T_1,T_2, $T_{1\rho}$ and NOE build-up rates of ^{15}N and ^{13}C nuclei order parameters and correlation times were derived to describe the dynamic behaviour of molecular parts. In particular the dynamics of the protein backbone and of amino acid side chains have been analysed. In general the motional parameters correspond to the structural features obtained with accurate ^1H-^1H distances and dihedral angle constraints. They also correspond to the motional parameters extracted from molecular dynamics simulations.

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COMPARING IRON AND MANGANESE SPECIFITY OF THE ACTIVE CENTER OF SUPEROXIDE DISMUTASE OF P. SHERMANII.

C. Scherk, M. Schmidt, H.-F. Nolting, B. Meier and F. Parak

The Superoxide Dismutase (SOD) from the anaerobic bacterium P. shermanii is expressed with different metal atoms which are built in at the center of the active site. Remarkably, the forms with either an iron or a manganese atom show a comparable enzymatic activity.
The enzyme was investigated by EXAFS spectroscopy. It was shown previously that the coordination sphere of the ferric SOD increases with rising pH. The coordination changes from five ligated in a trigonal bipyramidal shape at pH 6.4 to six ligated, tetragonal bipyramidal at pH 7.8. Presumably, the addition of a second solvent molecule explains this increase in coordination number and the decrease of the enzymatic activity at high pH.
The manganese containing form does not show this pH dependence. The enzyme is five coordinated from pH 6.4 to pH 9.3 with only slight changes in the radial distances of the coordinating atoms. Investigation of the XANES region suggests that the manganese is in the 2+ state. The addition of azide does not change the EXAFS spectrum which means that the ligand is not bound to the manganese ion in this oxidation state. Also the addition of dithionite neither changes the oxidation state of the manganese nor does it change the coordination.
In its native form the SOD of this enzyme apparently exists in either of the two postulated steps of the catalytic cycle: the iron containing species is predominantly in the oxidized, the manganese containing species in the reduced form.

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EFFECT OF INTRINSIC PROTEIN-LIPID INTERACTIONS ON STRUCTURAL AND FUNCTIONAL PROPERTIES OF Na,K-ATPase.

E. Schick[1], E. Grell[1], M. Mutz[2] and E. Marti[3].

The interaction of nonionic polyoxyethyleneglycolalcylether detergents such as $C_{12}E_8$ with purified renal and dogfish Na,K-ATPase leads to a transition from open membrane fragments to mixed micelles. During this process, enzymatic activity remains stable, although calorimetric studies (DSC) show a marked decrease of the denaturation temperature of the protein, which is indicative for a structural change. The interaction of Na,K-ATPase located in different intrinsic environments with K^+ is investigated by labeling the protein covalently with FITC as well as with Na^+ in the presence of the potential-sensitive dye RH-421, which incorporates nearby the enzyme in membraneous or micellar structures. Fluorescence spectroscopy measurements including precision titrations were carried out to determine binding affinities and stoichiometric coefficients. In the case of K^+-binding to membrane-bound Na,K-ATPase, two cations are bound per enzyme unit with binding constants around 10^5 M^{-1} for the renal and 10^4 M^{-1} for the dogfish enzyme. Binding affinity and stoichiometry remain nearly uneffected in the mixed micellar state. ATP-dependent phosphorylation of Na,K-ATPase in its membrane-bound as well as in its mixed micellar state in the presence of RH-421 can be detected by fluorescence spectroscopy. Whereas K^+-binding is not much dependent on the nature of the local lipid environment, the selective binding of Na^+ to the nonphosphorylated enzyme exhibits different properties, as concluded from spectrofluorometric titrations. In the membrane-bound state, Na^+-binding is also characterized by a 2:1 stoichiometry with binding constants around 10^3 M^{-1} to 10^4 M^{-1}. These Na^+-binding properties are markedly changed by more than one order of magnitude upon the transfer of the enzyme to the mixed micellar state. This result raises the question whether the structural changes, observed by DSC, affect the selective binding of Na^+ in the micellar state.

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1-113

THE STRUCTURE OF THE AZIDE COORDINATED SUPEROXIDE DISMUTASE (SOD) OF P. SHERMANII.

M.Schmidt, C. Scherk, B. Meier, F. Parak.

The cambialistic SOD of P.shermanii exhibits similar activity with iron and manganese in the same protein moiety. Both structures were solved recently to a resolution of 1.6 Å and 1.9 Å respectively by X-ray structure analysis [1]. Surprisingly, no structural differences between the Fe- and the Mn-SOD were visible. The central metal is 5-fold coordinated by three histidines, an aspartate and a water molecule or a hydroxide ion. Consistent structures were derived form EXAFS investigations performed on both metal forms at pH 6.4 [2]. In the presence of azide the SOD changes its coordination number from 5 to 6. This was also shown by EXAFS and results in a competitive inhibition of the SOD. It can be assumed that azide binds at the same site as the substrate O_2^-. Crystals of the Fe-SOD were soaked 4 days with increasing amounts of azide at pH 6.1. The final concentration was 30 mmol/l. A data set was collected to a resolution of 1.7 Å. In the refinement the structure of the native Fe-SOD including all X-ray visible water molecules was used as a start model. The final R-value was 15.4 %. The position of the azide appeared in a difference fourier map at a contour level of 4.0 σ. The first nitrogen atom of the azide binds to the iron at a distance of 2.28 Å. This hexa coordinated configuration is in consistence with our EXAFS investigation. The third atom of the azide is bound at a distance of 2.63 Å to the N_ϵ of His75 which also coordinates the iron. An additional water molecule is bound to the O_η of Tyr35 (3.38 Å) and the N_δ of His31 (3.15 Å). These residues form a channel through which the substrate is assumed to approach the active center. Both, the blocking of the channel and the binding of the azide could effectively inhibit the SOD activity. [1] Schmidt, Meier, Parak (1996) JBIC 1:532-541; [2] Scherk, Schmidt, Nolting, Meier, Parak (1996), Eur. Biophys. J 24:243-250

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1-114

DIFFERENT CONFORMATIONS OF TRP57 IN NSCP CALCULATED IN CHARMM22. A.Sillen , T.Braem and Y.Engelborghs

Sarcoplasmic Ca^{2+}-binding proteins (SCP) are one of the major subfamilies of EF-hand Ca^{2+}-binding proteins (Moncrief et al.,1990). SCP from the sandworm *Nereis diversicolor* is a single polypeptide chain that contains 174 amino acid residues and has 4 EF-hands (Mw=19485 D) (Collins et al.,1988). The second EF-hand is non-functional because of amino acid changes in the Ca^{2+}-binding loop. The high resolution X-ray structure of Nereis SCP was determined by S.Vijay-Kumar et al. (1992). This structure was used as starting structure for our calculations, it contains the 3 Ca^{2+} ions and no water. This structure was energy minimised. From this structure the χ_1 and χ_2 angles of Trp57 were set at different starting values, a short energy minimization was done to relax the Trp conformation, then the system was warmed up to 300 K and equilibrated . Then a normal molecular dynamics simulation of 50 ps was done to monitor the χ_1 and χ_2 angles of Trp57. It appears that Trp57 can stay in certain distinct conformations during the 50 ps and flips from one conformation to the other in other regions of the χ_1, χ_2 plot. This means that there are multiple stable conformations of Trp57 in NSCP. These different conformations could explain the multiple fluorescence lifetimes found for most single Trp-proteins. NSCP contains 3 tryptophans, the mutant with only Trp57 present has been made and measured, and also shows multiple fluorescence lifetimes.

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1-115

SOLUTION CONFORMATION OF THYMOSIN β4 AND MUTANTS. CORRELATION WITH BIOLOGICAL ACTIVITY. C. Simenel, C. Ampe, L. Van Troys, M. Delepierre and J. Vandekerckhove.

Thymosin β4, a polypeptide with 43 amino-acid residues, is involved in the regulation of actin polymerization. It is the major cellular actin-sequestering peptide shifting the polymerization equilibrium from filamentous to globular actin.

It has been shown (Vandekerckhove and coll.) that the N-terminal part of thymosin β4 (residues 1 to 24) is the smallest fragment able to reproduce the wild type sequestering activity. A stable helix covering residues 4 to 16 has already been established by NMR, thus, thymosin β4 mutant peptides were synthesized. So it was demonstrated that the residues L17, K18 and K19 establish specific contacts with actin, and that they are necessary to maintain a correct binding area with the actin. Mutations which affect the integrity of the N-terminal helix result in weaker actin binding and a decrease in the actin monomers sequestering activity. It was suggested that the distance between the N-terminal helix and the three actin binding residues was important to make contacts with the surface of the actin molecule.

We studied by NMR the solution structure of different mutants (S15A,S15AS, L17A and K11P) in a mixture of water-trifluoroethanol and compared with the structure of thymosin β4 obtained in the same experimental conditions, in order to establish a correlation between the structural modifications (N-terminal helix length an stability) and the activity (actin binding and actin monomers sequestering). In NMR, the length and the stability of the helix were determined by the measurement of α protons secundary chemical shifts. These experimental results are in agreement with predictions made with the algorithm AGADIR.

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SOLUTION STRUCTURE OF *Ace*-AMP1, A POTENT ANTIMICROBIAL PROTEIN EXTRACTED FROM ONION SEEDS. STRUCTURAL ANALOGIES WITH PLANT NONSPECIFIC LIPID TRANSFER PROTEINS. P. Sodano, S. Tassin, W.F. Broekaert, D. Marion, F. Vovelle and M. Ptak

Since the initial characterization of thionins in 1972, several antibiotic proteins have been isolated from various plants such as β-1,3 glucanases, permatins, ribosome inactivating proteins, cysteine rich antimicrobial peptides, plant defensins, albumin and several non specific lipid transfer proteins (nsLTP). Ace-AMP1 is a recently characterized protein extracted from onion seeds which displays a potent activity against bacteria and fungi. This protein synthesized with a 20 amino-acid propeptide is vacuolar. Its primary structure displays a high percentage of identity with maize nsLTP which structure has been recently resolved by NMR and X-ray diffraction. This last protein which transfers lipids between two membranes *in vitro*, is supposed to be involved in the transfer of cutin monomers and to participate to the plant defense. Its main structural feature is the existence of a long hydrophobic channel running through the molecule which is the binding site of lipids. The solution structure of Ace-AMP1 was established from 1300 distance constraints derived from 1H NMR data. It involves four helices which wind on a right handed super-helix as nsLTP structures. Two aromatic residues belonging to the H4 helix and the C-terminal segment point towards the protein core and plug the hydrophobic channel. The absence of any long and continuous internal hydrophobic pocket may explain why this protein is not able to transfer lipids between membranes.

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SIMULATION OF FUNCTIONAL CONFORMATIONAL PATHWAY IN DOMAIN III OF ANNEXIN V, J.Sopkova, S. Fischer, A. Lewit-Bentley and J. C. Smith.

Annexins form a distinct family of calcium binding proteins. All annexins contain a four- or eight-fold repeat of 70 residue sequence domain that are highly conserved, together with a more variable N-terminal segment. All crystal structures of annexins solved to date show that the sequence domains correspond to structural domains. Each structural domain, numbered I to IV, consists of five α-helices (A to E) and contains generally one principal calcium binding site. The calcium ion is bound to carbonyl oxygens of the loop connecting helices A and B and to a carboxyl of a negatively charged amino-acid side chain (Glu or Asp) some 40 amino acids downstream. It was shown that as long as the calcium ion is not bound in domain III in annexin V, the loop connecting helices A and B lies in the centre of the antiparallel helix bundle. The unique tryptophan of annexin V (Trp 187), which lies at the extremity of this loop, is buried in the interior of the protein. When the principal calcium site in domain III is formed in the presence of high calcium concentrations, significant structural changes in this domain are observed. The A-B loop moves in a direction parallel to the axis of helices A and B, thus bringing Trp 187 onto the surface of the protein. We have used the crystal structures of both forms of annexin V to model an adiabatic pathway of the observed conformational change in domain III using the conjugate peak refinement method, and molecular dynamic simulations. We have calculated the pathway using different solvatation corrections. Depending on the solvatation model used, we observe significant differences both in the energy barriers and in the intermediate conformations along the pathway.

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2-NAPHTHOL: A SPECTROSCOPIC TOOL FOR SOME LIPOCALINS. P. Cavatorta*, R. Pagani, G. Sartor, R.T. Sorbi* and A. Spisni.

Members of the lipocalin family are structurally characterized by a β -barrel made of eight-stranded antiparallel β-sheets forming a hydrophobic pocket that accommodates small organic molecules. Another related, but distinct, family of ligand binding proteins IS the fatty-acid binding protein family (FABP) whose β-barrel is ten-stranded.

A detailed knowledge of the molecular mechanism associated to the binding of small ligands to the protein hydrophobic pocket could be quite useful. In fact, it can guide protein engineering work to devise mutated protein where the specificity, affinity and release rate are properly modulated.

2-naphtol, a small hydrophobic molecule, has an emission fluorescence spectrum characterized by two well resolved peaks at neutral pH, one for the protonated form and the other for the unprotonated one.

We have been using these spectroscopic features to characterize the binding properties of three different proteins: two lipocalins such as the Major Urinary Proteins (MUP) and the Odorant binding Protein (OBP), and one FABP such as the Retinol Binding Protein II (RBP-II) that naturally bind distinct molecules.

The results indicate that : i) 2-naphtol binds only to the two lipocalins and not to RBP-II ; ii) only the protonated form of 2-naphtol binds to both lipocalins ; iii) 2-naphtol competes with the natural ligands of MUP for the same binding site.

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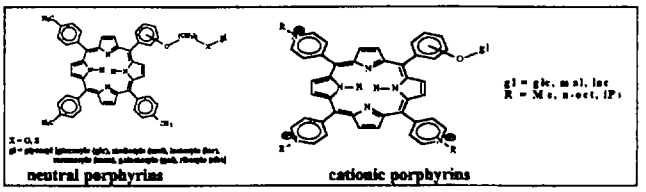
UV-VISIBLE AND FLUORESCENCE STUDIES OF NEW GLYCOSYLATED PORPHYRINS DERIVATIVES. M.Spiro[1], O.Gaud[2], V.Sol[2], I.Sylvain[2], C.Kaldapa[2], B.Négroni[2], R.Granet[2] and P.Krausz[2].



In connection with our research program on glycosylated porphyrins, we report here the synthesis and absorption and emission properties of neutral O and S glycosylated porphyrins, monomeric and dimeric cationic porphyrins. Their linkage with sugar moieties are of great importance because the carbohydrate increases water solubility, membrane interaction and specific receptor targeting in cancer photodynamic therapy. An analysis by uv-visible (splitting of Soret band at 420nm) and by fluorescence (loss of fluorescence intensity) show a specific stacking phenomenon for the neutral glycosylated porphyrins. Such phenomena are not observed for cationic porphyrins. These results have been extended to « peptidic » glycosylated porphyrins. An interpretation is given in term of the exciton theory. A dipole calculation let us suggest a shifted face to face structure for the aggregates.

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STRUCTURE AND DYNAMICS OF BACTERIORHODOPSIN STUDIED BY SITE DIRECTED SPIN LABELING. T. Rink, O. Burlon, M. Müller, M. Pfeiffer, J. Riesle, D. Oesterhelt, K. Gerwert, J. Schwemer and H.-J. Steinhoff

Site-directed mutagenesis allows the introduction of spin labeled side chains at nearly any desired site in a protein by using cysteine substitution mutants and selective attachment of nitroxide spin labels. The line shape of the continuous wave electron paramagnetic resonance (EPR) spectra contains information about the secondary and tertiary structure of the protein in the vicinity of the nitroxide side chain. A series of bacteriorhodospin cysteine mutants was labeled with a methane-thiosulfonate spin label. The structure and the structural changes of the cytoplasmic EF loop and in the vicinity of positions 46, 53, 89 and 93 during the photocycle and after the bacteriorhodopsin-bacterioopsin transiton were studied. The hyperfine tensor determined from EPR experiments at low temperatures provided the chemical potential of water in the putative proton channel in bacteriorhodopsin and bacterioopsin. Time resolved EPR spectroscopy revealed characteristic conformational changes of the cytoplasmic loops, in the vicinities of asp 96 and the retinal during the photocycle. A global fitting procedure of optical and EPR data showed that these conformational changes appear during the M1 to M2 and M2 to N transitions. These conformational changes differ in a characteristic way from the structural changes found for the bacteriorhodopsin-bacterioopsin transition. The interpretation of the EPR spectral line shapes was assisted by a new method for the simulation of EPR spectra of spin-labeled proteins that explicitly includes the protein structure in the vicinity of the attached spin label.

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1-121

THE EFFECT OF HELIX HELIX INTERACTIONS ON THE STABILITY OF TRANSMEMBRANE HELICES. J. Sturgis[1], B. Robert[1] and E. Goormaghtigh[2].

The aggregation of transmembrane helices is beleived to represent a step in the assembly of integral membrane proteins. We have investigated the peptide bond stability in the light-harvesting complex from *Rhodospirillum rubrum*, both in the intact form and after various degrees of dissociation. In this study we have used attenuated total reflectance FTIR spectroscopy to measure hydrogen/deuterium exchange rates in protein films. Due to our reasonable structural knowledge of this protein it has been possible to assign the various kinetically distinguishable groups of exchanging protons to different groups of amino acids in the three dimensional structure. We have further examined the evolution of the exchange rates during the stepwise dissociation of the protein from an oligomer containing 32 helices, via a tetramer to individual transmembrane helices in detergent solution. Previous authors have often failed to measure exchange rates for peptide protons in transmembrane helices. Our observation of measurable exchange rates in the transmembrane helices of the intact protein, is probably due to increased deuterium oxide accessibilty in detergent solution (compared to in the membrane). During dissociation we observe an appreciable increase in the exchange rates associated with the transmembrane helices. Though a certain proportion of this increase can be attributed to increase in deuterium oxide accessibility, detergents being more permeable than protein, there remains a proportion of this increase in exchange rate that we attribute to transmembrane helix destabilisation in the dissociated state. This observation would suggest that in addition to the entropic effects associated with the loss of side chain freedom during helix-helix aggregation there is in addition an entropic contribution from the stabilisation of the transmembrane helix backbone structure.

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1-122

PROTON NMR STUDY OF DENATURATION OF BOVINE SERUM ALBUMIN. A.Sułkowska

The thermal unfolding of bovine serum albumin in D_2O has been studied by proton NMR spectroscopy. All the spectra were taken with Bruker DPX 400 MHz spectrometer at temperature range 298-343K. The concentration of BSA solution in D_2O was 0.5M. Chemical shifts in ppm were measured with respect to a HDO signal 4.80 ppm.

Increase of temperature causes a transition from the folded to unfolded form of the protein. The first classification was to assign a resonance to a hydrogen attached to a carbon atom. -OH and -SH protons are not observed because their solvent exchange is too rapid.

Assignment of aliphatic (0.91-2.93 ppm at temperature 298K) and aromatic (6.30-7.15 ppm) proton resonance has been made with reference to the amino acids spectra. The methyl group resonance from a particular amino acids is uniquely characterized by the observed multiplet structure. The aliphatic CH and CH_2 resonance are overlaping and having complex multiplet structure.

The spectra of adenine-BSA complexe in D_2O at 298, 313, 323,333 and 343K were compared with the spectra of BSA.

A marked temperature dependence has been observed for the chemical shifts of purine (adenine) protons ($\Delta\delta = \delta_{343} - \delta_{298} = 0.53$ and 0.61 ppm for H-8 and H-2 protons respectively). lesser effect - for the chemical shifts of the aliphatic protons ($\Delta\delta = 0.44 - 0.53$ ppm) and the least - for the aromatic protons of BSA ($\Delta\delta = 0.41$ ppm).

Deshielding does not appear to be typical for the temperature effect on the resonance line. Chemical shifts of aromatic protons do not vary continuously with temperature. It seems that the adenine molecule forming at low temperature complex with BSA molecule, stabilizes the conformation of macromolecule at high temperature.

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1-123

STRUCTURE OF A DMPG/WHEAT NS-LTP COMPLEX FROM NMR DATA. **D. Sy, A. Caille, G. de Person, Y. Ohyama, D. Marion, M. Ptak, P. Sodano.**

Plant non specific lipid transfer proteins ns-LTPs are small basic proteins known to be capable of transferring phospholipids between two membranes *in vitro*. Several structures of plant ns-LTPs have been determined by X-ray diffraction and in solution. Three complexes with fatty acid, phospholipid and CoA have already been described. Here we report the first study on complexation of the wheat ns-LTP with a double acyl chain lipid (dimyristoyl-phosphoglycerol, DMPG). The stucture of the complexed protein has been built with the DIANA program and NMR distance list extracted from two NOESY experiments. The protein global fold is similar to the free protein one. Observation of NOEs between the protein and the lipid, *i.e.* Arg44 and His35 residues and the glycerol moiety, confirms that the protein entrance is located on the L2 loop. However, significant differences are observed in the hydrophobic core of the protein and in the C-terminus ; the Tyr79 residue, which seems to play a key role in the lipid complexation, is greatly affected and its side-chain is rotated. These differences could be related to the mechanisms of lipid capture or release as the protein molecule is adsorbed on a membrane.

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A CALORIMETRIC STUDY OF THE BINDING OF LISINOPRIL TO BOTH APO- AND HOLO- ANGIOTENSIN CONVERTING ENZYME FROM BOVINE LUNG. R. Téllez-Sanz; L. García-Fuentes and C. Barón

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a zinc-metallopeptidase consisting of two homologous domains, each containing a putative metal-binding site [1]. ACE is a target for antihypertensive drugs, such as lisinopril (furanacryloyl-L-phenylalanil-glycylglicine).

High sensitivity titration calorimetry was used to measure changes in enthalpy for binding of lisinopril to both apo- and holoenzyme in cacodylate buffer at pH 7.5 and 25°C. The enzyme was purified to homogeneity as was described by García-Fuentes *et al.* [2].

Lisinopril binds to two sites of holoenzyme monomer. The ΔH obtained was 42.48 kJ·(mol of monomer)$^{-1}$ for the binding of lisinopril to the holoenzyme, while this value was reduced to 23.8 kJ·(mol of monomer)$^{-1}$ for the apoenzyme. The positive ΔS and ΔH values obtained for the holoenzyme can be explained by hydrofobic effects and electrostatic interactions.

We conclude that lisinopril binds to two sites *per* monomer of ACE. The binding process is entropically driven at 25°C and electrostatic interactions between Zn^{2+} and lisinopril greatly enhance the affinity of ACE for this ligand.

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1-125

pH and Ionic Strength Control on the Temperature-Dependent States of Wild and Cloned DNA-Binding Protein Sso7d from *Sulfolobus solfataricus* R.Todorova, B.Atanasov, S.Knapp, A.Karshikoff and R.Ladenstein

The acidic pH- and ionic strength-dependence of thermodynamic functions of wild DNA-binding protein Sso7d from *Sulfolobus solfataricus* as well as its cloned (c-Sso7d) in glycine and phosphate buffers was studied by means the adiabatic scanning calorimetry. From ΔHcal(pH) and Td(pH) experimental data and the sharpness of transition (∂Td/∂TpH) the difference in proton binding was estimated. It was found that a single group non-cooperative ionization with apparent pKa=3.25 rule thermal unfolding of two different (protonated and unprotonated) forms. Because ΔH^o is obtained to be pH-independent the changes in stability (quantatized by ΔG^o) originate from changes in entropy terms. The apparent pKa "at satured" phosphate concentration decrease with 0.5 units which determine free energy of transfer from zwiterionic glycine to monopole charged phosphate to be small as 0.7 kcal/mol. The min/max values of ΔG^o in neitral and acidic pH also changed in correlative mode that express reversibility of the system. Ionic strength dependence at pH 6.0 and 1.5 - typical for the two pH-dependent D-states are wery different (in amplitude and sign of changes). This reflect major property of denatured (unfolded) state to be "closed" (Dc) at neutral or weak acidic pH and "open" (Do) at acidic pH.

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ABOUT THE PHOSPHORESCENCE OF α-CRYSTALLIN

F. Tölgyesi, B. Ullrich and J. Fidy

α-crystallin is the major protein of vertebrate eye lens. Besides of maintaining the appropriate optical characteristics of the lens it probably plays a more complexed role as a heat-shock protein also in other tissues. Our aim was to characterise the phosphorescence of α-crystallin in order to have a tool for monitoring conformational changes in the protein molecule.

Room temperature phosphorescence measurements revealed the usual emission spectrum with a maximum at 442 nm and a 3-component decay curve with lifetimes ranging from 23 ms to 500 ms. The three components were interpreted as the three tryptophan residues in subunit A and B of the molecule. The lifetime values and also the calculated hydropathy profile show moderately hydrophobic microenvironments for two residues and somewhat more hydrophobic surrounding for the third one.

Low temperature (10-250 K) measurements revealed no significant heterogeinity in tryptophan environments: no splitting occured in the peaks of the emission spectrum, which would be a sign for tryptophans of very different burial; the glassy transition of the protein is found around 200 K.

Probing the accessibility of tryptophans with acrylamide quenching experiments we found slightly higher quenching constants ($2\text{-}9\times10^3$ M^{-1} s^{-1}) for the shorter lifetimes and a lower value (3×10^2 M^{-1} s^{-1}) for the longest lifetime which supports our previous assumptions.

The temperature dependence of the phosphorescence lifetime was measured between 10 and 50 °C. Arrhenius plots exhibited discontinuities at around 28 °C for all of the components, showing probably reversible conformational changes in the protein.

Aggregation, initiated by heating the sample to 55 °C and stabilized with $CaCl_2$, altered the phosphorescence of α-crystallin.

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A TIME-RESOLVED FTIR DIFFERENCE SPECTROSCOPY STUDY OF THE SR CA^{2+}-ATPASE: KINETICS OF THE HIGH AFFINITY CALCIUM BINDING AT LOW TEMPERATURE

A. Troullier, K. Gerwert* and Y. Dupont

We have used time-resolved FTIR difference spectroscopy to characterize the amplitude, frequency and kinetics of the absorbance changes induced in the IR spectrum of the sarcoplasmic reticulum Ca^{2+}-ATPase by calcium binding on the high affinity transport sites. DM-Nitrophen was used as caged-calcium compound to trigger the release of calcium in the IR samples.

Calcium binding on the Ca^{2+}-ATPase induces the appearance of spectral bands in difference spectra which are all absent in the presence of the inhibitor thapsigargin. Spectral bands above 1700 cm^{-1} indicate that glutamic and/or aspartic acid side chains are deprotonated upon calcium binding whereas other bands may be induced by reactions of asparagine, glutamine and tyrosine residues. Some of the bands appearing in the 1690-1610 cm^{-1} region arise from modifications of peptide backbone carbonyl groups. The band at 1653 cm^{-1} is candidate for a change in an α helix whereas other bands could arise from modifications of random, turn or β sheet structures or from main chain carbonyl bonds playing the role of calcium ligands. Only a few residues are involved in secondary structure changes.

The kinetic evolution of these bands was recorded at low temperature (- 9 °C). All bands exhibited a monophasic kinetics of rate constant 0.026 s^{-1} which is compatible with that measured at the same temperature in suspension of SR vesicles by intrinsic fluorescence of the Ca^{2+}-ATPase.

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THE REFLECTOMETRY STUDIES OF SPATIAL STRUCTURE OF PROTAMINE

V.Tsygankov, L. Ochotnikova

INTRODUCTION: Protamines are low molecular weight basic proteins which have been found associated with DNA in the sperm head from different animal species. The complex found in the sperm nuclei contains both DNA and protamine. The structure of this complex is not known, althoug several mutually exclusive models have been proposed. Since the DNA conformation is known as to be the beta-form, however, the possible approach to the structural problem is elucidation of protamine's molecular conformation in solution by Time Domain Dielectric Spectroscopy (TDDS) method.

MATERIALS AND METHODS: The protamines were from SIGMA Chemicals and used without further purification. The concentration of the sample solutions was 2 %. Measurements were made by automatic TDDS spectrometr.

RESULTS AND DISCUSSION: The behaviour character of temperature dependences of the dielectrical relaxation times, dipole moment and effective volume of the protamine macromolecule sharply differs from the ones expecting for globular protein and can't described by the globular state of protamine. There are, apparently, protamones as statical ball in aqueous solution. There is, as well, apparently, the point Flory for the protamine macromolecule and the transition to the Gauss ball in the temperature region from 50 to 60 C. In this temperature region, the forces of attraction and repulsion are compensated and the behaviour of protamine macromolecule can be described by ideal Gauss ball.

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STRUCTURAL EFFECTS OF INHIBITOR BINDING IN HIV1 PROTEASE.

B. Ullrich[1], F. Tölgyesi[1], Z. Szeltner[2], L. Polgár[2] and J. Fidy[1]

We studied the effect of inhibitor-binding on the structure of HIV1 protease by its luminescence parameters such as the fluorescence excitation and emission spectra, the quenching of fluorescence with acrylamide and iodide and the temperature dependence of low temperature phosphorescence.

We also investigated the structural changes in the apoenzyme and enzyme-inhibitor complex during denaturation using urea as denaturing agent.

The two tryptophanes of the native apoenzyme were found differently accessible to the solvent and their fluorescence is distinguishable based on the bimolecular quenching constants (k_q) of the two lifetime components.

The binding of the acetyl-pepstatin inhibitor makes the protein structure more compact and stable, as shown by the decreased quenching constant of the τ_1 component, however, there is no drastic change in the neighbourhood of tryptophanes is indicated by other fluorescence parameters. The stabilizing effect of the inhibitor-binding is also confirmed by the different temperature dependence of phosphorescence in case of the apoenzyme and the enzyme-inhibitor complex.

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DYNAMICS OF ALCR(1-60)* FREE AND BOUND TO AN OLIGONUCLEOTIDE TARGET FROM 15N NMR RELAXATION STUDIES. C. van Heijenoort, G. Goussard, R. Cerdan and E. Guittet.

The DNA binding domain, alcR(1-60)*, of the transcription regulator ALCR belongs to the family of Zinc binuclear clusters exemplified by GAL4. It exhibits an unusual 16 a.a. insertion, between the third and fourth cystein. It also shows a striking strong affinity as a monomer for half site DNA targets, leading to an alcR(1-60)*-DNA complex in slow exchange in the NMR chemical shift time scale. Dynamic studies give valuable information about the modification of the flexibility of the protein upon binding. The ^{15}N NMR relaxation parameters T_1, T_2 and heteronuclear ^1H->^{15}N nOes were measured on the free and bound protein. The spectral densities of the backbone and arginine side chains NH bonds were calculated at three frequencies, 0, ω_N, and around $\omega_H+\omega_N$, using a reduced matrix approach, and were analysed in term of motions using various approaches.

The most interesting results concern the εNH bonds of arginines. Their motions are globally more restricted in the bound state than in the free state. The εNH bond of arginine 28, already hindered in the free state, appears more restricted in the bound state. This arginine is located in the extended sequence. The εNH bond of arginine 20, very flexible in the free state, is completely blocked upon binding. It is located between the second and third cystein, a region known to make direct contacts with the DNA in the case of GAL4.

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THE INFLUENCE OF THE ISOLATION CONDITIONS ON THE QUATERNARY STRUCTURE AND INTERACTION PARAMETERS OF BOVINE α-CRYSTALLIN. J.Vanhoudt ,T.Aerts and J.Clauwaert.

α-Crystallin is the main protein component in the eye lens fiber cells. Its solution properties combine a high refractive index, due to its high concentration, and a low light scattering power.

The general expression for light scattering intensity $R_p(k)$ is given by

$$\frac{Kc}{R_p(k)} = \frac{1}{P(k).M_w.S(c,k)}$$

with S(c,k) the solution structure factor, which takes into account the spatial distribution of the solute particles, and P(k) the particle form factor. At low concentrations and particles small relative to the wavelength of the incident beam, the above equation becomes:

$$\frac{Kc}{R_p(k)} = \frac{1}{M_w^0} + 2Bc$$

The molar mass M^0_W and second virial coefficient 2B, which quantifies the solute-solute and solute-solvent interactions, can also be determined from sedimentation equilibrium studies. The quaternary structure and the second virial coefficient of α-crystallin are still a matter of discussion. Molar mass values from 300.000 to 800.000 g/mol and second virial coefficients from the hard sphere value up to values 10 times higher, suggesting a strong repulsive interactions, have been published. We have studied the influence of the isolation temperature on M^0_W and 2B. We indeed find important changes as a function of temperature, which does explain some discrepancies in published data and shed some light on the chaperone activity of α-crystallin. In our hands, both methods give consistent and accurate values of the molar masses whereas the 2B values are more contaminated by experimental noise.

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ROLE OF SEQUENCE REPETITIVE MOTIVES OF THE CARBOXY TERMINAL MODULE OF LytA AMIDASE FROM *S. pneumoniae*. J. Varea, M. Gasset, J. Laynez, J.L. García, J.L. Saíz, C. López-Zúmel, P. Usobiaga and M. Menéndez.

Bacterial autolysins are endogenous enzymes that specifically cleave covalent bonds in the cell wall. These enzymes present both substrate and bond specificities. LytA amidase, the major autolysin from *S. pneumoniae*, has a modular organization; i.e., the N-terminal module, responsible for the catalytic function, and the C-terminal module, involved in the recognition and attachment to the cell wall. The latter consists on six repeated sequences of 21 a.a. (motives p1-p6) and a tail 11 a.a., and specifically binds to choline residues of the teichoic acids. The C- terminal region presents two different classes of choline binding sites. Saturation of the higher affinity sites shifts the monomer↔dimer equilibrium of the enzyme towards the dimeric form.

The aim of the present work has been to characterize the structural and thermodynamic implications of the repeated sequence motives of the C-terminal module, by studying the structural organization, the choline binding capacity and the choline-modulated autoassociation of four mutants produced by serial deletions of p6 and p5 units, including or not the terminal tail (P6, P5, P5c and P4 amidases) by circular dichroism, Fourier transformed infrared techniques, differential scanning calorimetry and sedimentation equilibrium. The truncated mutants present minor differences in their relative content in secondary structure with LytA amidase, but ligand affinity, choline-regulated autoassociation and thermal stability are extremely sensitive to any supression. The p6 motif and the terminal tail are involved in regulation of the autoassociation process and are essential for preferential binding to dimeric amidase. Major binding to the monomeric form in all the truncated mutants results in a significant reduction of choline affinity. The higher destabilizations of the C-terminal module result from deletion of the tail (P6 amidase) and the p5 motif (P5 →P4 amidase).

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PROPERTIES OF CERULOPLASMIN FROM SERUM OF A PATIENT WITH WILSON DISEASE. V.B. Vassiliev[1], V.De Filippis[2], E.T. Zakharova[1], M.V.Neuymina[1], M. Beltramini[3], V.S. Gaitskhoki[1], A. Fontana[2] and B. Salvato[3].

Ceruloplasmin (CP) from serum of a patient with Wilson disease (WD) had no oxidase activity assayed with either p-phenylene diamine or o-dianisidine, contained trace amounts of copper, but had the same Mr (132 kD) and immunological properties as the native CP of healthy donors. Gel-filtration (HPLC) of CP-WD showed that, moving much slower than apo-CP, its peak coincides with that of native CP. CP-WD absorbed neither at 610 nm, nor at 330 nm due to the absence of type I and type III copper ions. Its fluorescence was more intensive due to unquenching occurring with loss of copper, but the spectrum (λ_{exc}=295; λ_{emiss}=350nm) was similar to that of native CP. Excitation at 280nm revealed a small shoulder in the 300-nm region, pointing at the lack of Trp-to-Tyr energy transfer that normally occurs in holo- and apo-CP, and is disturbed only in course of denaturing. Even 30 min of incubation of CP-WD with ANS resulted in a drastic increase of fluorescence as compared to ANS-holo- and ANS-apo-CP, which indicates that much more hydrophobic regions are exposed in CP-WD. Near- and far-UV CD spectra of CP-WD differed from those of holo- and apo-CP, pointing at partial loss of β-structure. Second derivative spectra of holo-, apo-CP and CP-WD were similar. CP-WD was almost uncleaved by "spontaneous" proteolysis in contrast to both holo- and apo-CP. When treated with trypsin CP-WD was rapidly cleaved into fragments with low Mr, while holo- and apo-CP were producing intermediate components. On account of the data obtained in biophysical and biochemical studies of CP-WD it can be concluded that this protein has an altered structure as compared both to the native and copper-depleted CP.

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MODELLING PATHWAYS OF α-CHYMOTRYPSIN ACTIVATION AND DEACTIVATION. B.Wroblowski[†], J.F.Diaz[†], J.Schlitter[‡], G.Verheyden[†] and Y.Engelborghs[†]*

The conformational change of α-chymotrypsin from an inactive, chymotrypsinogen like structure at high pH to an active conformation around pH 8.5 is used here as a model system to generate possible pathways for the transition by use of two different theoretical methods. One method, the 'targeted molecular dynamics' algorithm (TMD) [1] adds a constraint in the direction of the target to a molecular dynamics force field and gives two different paths, one for every direction of the reaction. The second method, the 'self penalty walk' algorithm (SPW) [2], refines an initially guessed path by minimizing the sum of the energies of its structures. Thus, starting from a linear path as first approximation, it produces a reaction coordinate of the transition. The structures of the TMD and SPW paths are similar only in the beginning while the middle part of the SPW path links the two TMD branches. The activation of α-chymotrypsin in the TMD path starts with a movement of loop VII (residues 215-225), pulling on loop VI (residues 186-194). Then the side chain of Met192 turns to the surface and Ile16 approaches Asp194 to form a salt bridge. In the TMD deactivation path loop VII also moves and pushes loop VI to the protein core. The Met192 side chain adopts three intermediate conformations, till the salt bridge Ile16-Asp194 is broken and loop VI rearranges to its final conformation. In the SPW pathway, both the formation of the salt bridge and the movement of Met192 happen simultaneously between two consecutive steps

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MAG-INDO-1 AS AN EXTRINSIC FLUORESCENT NON-COVANLENT PROBE FOR PROTEIN BINDING. J. Vigo, T. Vo-Dinh, T. Bunde, P. M. Viallet and J.M. Salmon.

Mag-indo-1 is a well known fluorescent probe, designed to complex magnesium but which has been demonstrated to bind to proteins using gel filtration techniques. Moreover such a binding occurs through a specific interaction with histidin residues. This interaction results in a specific shift of the fluorescence spectrum of Mag-indo-1 which was found independent of the nature of the protein. That makes it possible to quantify the interaction between the fluorescent dye and the protein using a convenient method of resolution of fluorescence spectra.

Interaction of Mag-indo-1 with egg white lyzozyme, a protein known for bearing only one histidin residue was first studied. Data obtained for the titration of lyzozyme with Mag-indo-1 are consistent with a 1/1 interaction with a Kd value in the range of that found for the interaction with free histidin.

Then interactions of Mag-indo-1 with Bovin Serum Albumin (BSA) or Human Serum Albumin (HSA) were studied. Data obtained for these titrations fit perfectly a model of allosteric interaction with four subunits which is consistent with the secondary structure of these proteins.

Moreover this binding was associated with a strong quenching of the fluorescence of the tryptophan residue(s) of BSA while the fluorescence of the tryptophan residue of HSA was not affected.

These findings suggest that Mag-indo-1 migth be used for probing protein 3D conformation and 3D conformation dependence on pH and temperature.

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PROTEIN CRYOFLUORESCENCE: THE INFLUENCE OF SOLVENT ON THE DYNAMIC TRANSITIONS AROUND 200K. M. VINCENT[¶], R. SARFATI[§], O. BÂRZU[‡], A.-M. GILLES[‡] and J. GALLAY[¶]

Thermal transitions affecting the local protein dynamics in two different regions of the cytidine monophosphate kinase, have been detected by measuring the excited state fluorescence lifetime of the single tryptophan residue (Trp31) and of a fluorescent competitive inhibitor (dADP-anthranyloyl, d-ADPA) as a function of temperature in cryogenic glycerol/water mixtures. These two fluorophores are localized in opposite regions of the protein: Trp31 is buried in a β-barrel while dADP-A is bound in the active site. The major excited state lifetime of Trp31 increases sharply upon decreasing the temperature down to 175-200K and then reaches a plateau value. This temperature range corresponds to that of the *vitrous transition* of the solvent *(Luyet et Rasmussen, 1968, Biodynamica 10, 167).* The thermal-induced variation of the dADP-A excited state lifetime is more complex. After a sharp increase, the lifetime becomes maximum for a temperature of 240K, decreases again and finally reaches a plateau at 150-160K. A similar trend is observed for the probe in the cryogenic solvent. The 240K temperature corresponds to the *melting transition* of the solvent mixture while the 150-160K point corresponds to the *vitrous transformation.* It appears therefore that the nucleotide binding site is extremely sensitive to the physical state of the solvent while, the Trp31 region is more protected. The physical state of the solvent is an important factor for the control of the dynamic state of the protein.

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1-137

SOLUTION STRUCTURE OF THANATIN, AN INSECT DEFENCE PEPTIDE WITH ANTIMICROBIAL PROPERTIES.

N. Mandard, P. Sodano, H. Labbe,J. M. Bonmatin, M. Ptak and F. Vovelle.

Thanatin is a 21 residues inducible defence peptide isolated from the hemipteran insect *podisus maculiventis.* It is the first, insect defence peptide to show at physiological concentrations an activity against Gram-positive and Gram-negative bacteria as well as against fungi. This peptide, is strongly cationic and shows a 6 residue basic loop stabilized by the disulphide bridge binding residue 11 to 18. An all D- and several truncated isoforms were synthesized and tested for antimicrobial and antigungal activity on several bacterial strains. A minimum motif consisting of the C-terminal loop and the flanking residues was shown to confer a detectable activity.

In order to draw structure-function relationships for this family of peptides, two-dimensional NMR spectroscopy and molecular modelling were used to study the conformation of native thanatin in aqueous solution. Except for the 6 N-terminal residues which are completely unstructured most of the structure is particularly well defined. Thanathin forms a β sheet including residues 9-13 and 16-21 and stabilized by several non canonical hydrogen bonds. This well defined region of the peptide correspond to the minimum motif showing a detectable activity against bacterial strains. This segment includes polar an basic residues which constitute the binding site of the peptide to the negative surface of the bacteria.

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STRUCTURAL AND HYDRATION CHANGES OF BACTERIORHODOPSIN PROBED BY NEUTRON DIFFRACTION.

M. Weik, Th. Hauß, N.A. Dencher, D. Oesterhelt and G. Zaccai

Bacteriorhodopsin (BR) is a transmembrane, light-driven proton pump. It is naturally arranged in a two-dimensional lattice within the Purple Membrane (PM), part of the cytoplasmic membrane of *Halobacterium salinarium*. Upon absorption of a photon by the light sensitive molecule retinal, which is covalently linked to the protein moiety, BR undergoes a series of photointermediates and pumps a proton from the cytoplasmic to the extracellullar part of the membrane. Structural changes between the Groundstate and the key photointermediate M have been reported for fully hydrated PM and are supposed to play a functionally important role in the proton pump mechanism. We performed neutron diffraction experiments at various hydrational states of PM, coupled to H_2O/D_2O exchange, in order to study these structural changes at different relative humidities (h.r.) and to localize functionally important water molecules within the PM. We observe structural changes between the Ground- and the M-state only above 60% r.h., even though formation of the M-state takes place at low r.h. as determined by absorption spectroscopy. The M-state is actually composed of two substates, connected by a non-reversible transition to ensure unidirectionality of proton transfer. On the basis of our experiments, we attribute the observed structural changes to the transition between these two M-substates. Difference Fourier analysis of diffraction data in H_2O and in D_2O revealed a similar number of water molecules in the proton pathway of BR in the Ground- and the M-state. We conclude that the observed structural changes are not coupled to an increase of the amount of water molecules in the proton channel, as it was originally assumed for functional reasons.

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1-139

ON THE TRANSPORT MECHANISM OF THE SUGAR SPECIFIC OUTER MEMBRANE PROTEIN LamB

M. Winterhalter, N. Saint, Y.F. Wang and J.P. Rosenbusch

The transport rates for maltotriose and maltohexaose through maltoporin and their variations with specific mutants were obtained from the spectral density of the current noise and were related to the recently published high resolution x-ray structure. Maltoporin, a sugar specific outer membrane protein was reconstituted into planar lipid bilayer. Titration with maltose sugar caused blocking of the channel observed by a reduction in conductance. The analysis of the time resolved conductances as a function of the sugar concentration yield the binding constant as well as the on and off-rate of the sugar binding. These rate constants are directly related to the sugar translocation. Various aromatic residues (W74, Y6, W420, W358, F227 and Y118) were mutated against alanine. The off-rate increased twice or 4 times by substituting the aromates in the inner part of the channel (Y6A, W420). A much stronger effect was seen for the on-rate which is at low sugar concentration directly proportional to the translocation rate. The latter was reduced by 100 for the inner aromates (Y6A, W420A, Y118A). Mutation of the aromates in the neigborhood (W74A, W358A) reduced the on-rate for maltohexaose by 10, whereas little effect was seen for maltotriose. As expected from the x-ray structure the outer aromate (F227A) seems to play only a minor role.

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1-140

DYNAMICS OF A GLOBULAR PROTEIN AS STUDIED BY QUASI-ELASTIC NEUTRON SCATTERING AND NMR.

J.-M. Zanotti[1], M.-C. Bellissent-Funel [1] and J.Parello [2]

Hydration has been shown to play a crucial role in protein function **[1]**. Effect of hydration on the dynamics of parvalbumin, a 11.5 kDa Ca^{2+}/Mg^{2+} binding globular protein, has been studied by incoherent quasi-elastic neutron scattering and ^{13}C solid-state NMR. Samples were hydrated powders of protein hydrated at different hydration level, from dry state to 0.65 g D_2O / g dry protein.

From the analysis of the quasi-elastic spectra, the elastic incoherent structure factor, which describes the time average spatial distribution of the hydrogen atoms of the protein, has been evaluated. It has been shown that for a level of hydration of 0.65 g/g, 30% of the protons adopts a diffusive motion which is limited to a sphere of 1.7 Å radius. The increase of the quasi-elastic signal, as observed in neutron scattering upon hydration, is interpreted as an increase of the local mobility of side chains protons, mainly of the hydrogen rich lysyl residues. This result is in agreement with a parallel study of solid-state natural abundance ^{13}C cross polarization and magic angle spinning NMR. The analysis of the mean square displacements of the hydrogen atoms, as deduced from neutron scattering, leads to the fact that, for low levels of hydration ($h \leq 0.30$ g/g), the Mg-loaded form of parvalbumin is characterized by a more constraint dynamics than the Ca-loaded form. This looks in agreement with x-rays crystallographic results that show that the substitution of Ca^{2+} by Mg^{2+} is characterized by a contraction of the hydrophobic core of the protein **[2]**.

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1-141

CRYSTAL POLYMORPHISM OF METHIONYL-tRNA SYNTHETASE FROM *E. COLI*.

G. Verdon, Y. Mechulam, L. Serre, T. Choinowski, N. Hervouet, J.L. Risler & Ch. Zelwer.

Methionyl-tRNA-synthetase (MetRS) belongs to class 1 aminoacyl-tRNA synthetases and to a sub-family which includes the hydrophobic non aromatic aminoacids. Some of them including MetRS are involved in editing activities against tRNA mischarging by non-cognate competitor aminoacids. The structure of an active fragment of MetRS (551 residus) has been solved partially by X-ray crystallography. That fragment crystallizes within two crystal forms (form I and form II) by seeding techniques. They exhibit different crystal habits and different stability when soaked in a methionine containing solution. Form II crystals are stable in the presence methionine, contrary to form I crystals which loss transparency and diffracting power. Diffusion of methionine into the form II crystals has been verified with ^{35}S methionine. Both forms grow in the same crystallization drop, but form II occurs less frequently and in smaller sizes. Furthermore, addition of methionine to the crystallization conditions leads to form II exclusively. The same results can be obtained with either ammonium citrate or ammonium sulfate as a precipiting agent. Data collections (2.8 Å resolution) carried out at 100 and 280°K, confirmed the existence of these two crystals forms which have the same space-group and slightly different cell parameters. The structure of the MetRS-met complex will allow an insight into the recognition site of this substrate and an analysis of conformation changes as well.

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2-1

THE CYTIDINE-RICH STRAND OF CENTROMERES AND TELOMERES: AN NMR INVESTIGATION OF INTER-CALATED DNA STRUCTURES. J.-L. Leroy, S. Nonin, X. Han, Phan A. T. and M. Guéron.

Telomeres and centromeres are nucleoprotein assemblies involved in chromosome and cellular replication. Their specialized, non-coding DNA includes short DNA repeats such as $CCCTA_2$ for vertebrate telomeres and $CCAT_2$ for human centromeres. Such sequences form intercalated duplexes of opposite orientations, each duplex consisting of parallel strands connected by hemi-protonated $C{\cdot}C^+$ pairs. This is the so-called i-motif, presently the only known nucleic acid structure involving systematic strand intercalation. It features short H1'-H1' and amino-H2'/H2" distances between protons of oppositely oriented strands, generating characteristic NOESY cross-peaks which reveal the intercalation topology.

We have shown that 4-times repeats of telomeric and centromeric sequences fold into an intramolecular i-motif. However the poor quality of the NMR spectra, presently unexplained, prevents the resolution of the structure. We approach this problem with related deoxy-oligonucleotides, selected for their good NMR spectra. The i-motif may be formed by a tetramer of a strand carrying one C-stretch, e.g. d(TCC), or by a dimer of a strand with two C-stretches, or by the intra-molecular fold of a strand with four C-stretches, as above. The stoichiometry is determined from the concentration dependence of the multimer-monomer equilibrium.

We have obtained the high-definition structure of an analog of a human centromeric sequence. Intra-molecular folding creates an i-motif core which is extended by stacking of loop residues, and is stabilized by base-pairing in and between the loops. The structure is stable up to pH 7.4.

The influence of the nucleotides between C-stretches is probed with strands containing two C-stretches. The dimer of d(5mCCTTTACC) has TTTA loops on either side of the i-motif core, whereas $d(5mCCTTTTCC)_2$ has the TTTT loops on the same side of the core. Symmetry is broken in $d(5mCCTCC)_4$, whose two duplexes are unequivalent, the thymidines being paired in one and unstacked in the other. The structure and kinetics of this macromolecular switch have been determined.

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2-2

KINETICS OF DNA HYDRATION. *V.P. Denisov, G. Carlström, H. Jóhannesson, K. Venu, and B. Halle.*

The hydration of the d(CGCGAATTCGCG) *B*–DNA duplex in solution was studied by nuclear magnetic relaxation dispersion (NMRD) of the water nuclei ^1H, ^2H, and ^{17}O, and by nuclear Overhauser effects (NOEs) in high-resolution two-dimensional ^1H NMR spectra. By comparing results from the free duplex with those from its complex with netropsin, water molecules in the "spine of hydration" in the AATT region of the minor groove could be distinguished from hydration water elsewhere in the duplex. The ^2H and ^{17}O relaxation dispersions yield a model-independent residence time of 0.9 ± 0.1 ns at 4 °C for five highly ordered water molecules in the spine, quantitatively consistent with the new NOE data. At 27 °C, the residence time is estimated to 0.2 ns, a factor 40 shorter than the tumbling time of the duplex. The NMRD data show that all water molecules associated with the duplex, except the five molecules in the spine, have residence times significantly shorter than 1 ns at 4 °C. There is thus no long-lived hydration structure associated with the phosphate backbone. In contrast to ^2H and ^{17}O, the ^1H relaxation dispersion is dominated by labile DNA protons and therefore provides little information about DNA hydration.

^2H and ^{17}O NMRD data from the d(TAGCGTACTAGTACGCT) *B*–DNA duplex show that the minor groove hydration is more labile than in the dodecamer (subnanosecond residence times at 4 °C). NMRD measurements have also been performed in deeply supercooled DNA solutions using an emulsion technique.

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2-3

NOVEL FOLDING PATTERNS IN RNA. NMR STUDIES OF AN RNA HAIRPIN AND PSEUDOKNOT. M Kolk, H.A. Heus, S.S. Wijmenga and C.W. Hilbers.

Progress in NMR methodology and the possibility to enrich RNA molecules in ^{13}C and ^{15}N isotopes have paved the way for in-depth studies of the structure of RNA molecules. In this presentation two examples will be discussed, i.e. the structure of an RNA hairpin, which is part of the catalytic site of the ribozyme present in the self-cleaving sequence of hepatitis-delta virus antigenomic RNA, and the structure of the pseudoknot formed in the 3'-end of TYMV RNA.

The hepatitis-delta virus hairpin constists of a stem closed by a seven-nucleotide loop. This loop is composed of six pyrimidines and one purine. Nevertheless, it turns out to have a stable structure, mainly supported by sugar hydroxyl hydrogen bonds and by base-base and base-phosphate stacking interactions. The folding in this loop is stabilized by some novel structural features, a reversed U-turn and a new non-Watson-Crick U.C base pair formed between the first and the last residue in the loop.

The heart of the structure of the TYMV pseudoknot is formed by a colinear system of three stacking helices, a pseudoknot and a stabilizing hepta-loop hairpin. A large number of NMR experiments on uniformly and selectively labeled samples were needed to determine the three-dimensional structure of the molecule. The central part of the molecule, the pseudoknot junction, is particularly interesting. The base pairing scheme in the double helical region as well as the non-helical base sequences around the junction are conserved in other plant viruses (in the tymoviral and tobamoviral RNAs) but are also found in the P4/P6 region of the main phylogenetic variant of the group I intron. We obtained a detailed picture of the interactions in the pseudoknot junction, which apart from base stacking interactions is stabilized by triple helix formation and sugar hydroxyl hydrogen bonds.

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2-4

MODELLING LARGE SCALE DEFORMATIONS OF DNA

R. Lavery, A. Lebrun and D. Flatters

DNA is a very flexible polymer and conformational changes are a vital part of its biological functioning. In particular, protein binding often induces deformations which can play a role in the specific recognition of target base sequences and in the preparation of further interactions. Molecular modelling can be used to gain insight into the mechanisms and the energetics of such conformational changes and has been applied by us to understanding major structural deformations such as those induced by the TATA box binding protein.

Our modelling involves molecular mechanics, using a simplified internal and helicoidal coordinate model of DNA, enabling extreme stretching, twisting and bending to be generated. The results obtained in connection with nanomanipulation experiments show unexpected links between mechanically and protein induced deformations. These results have been extended by molecular dynamics simulations which enable the detailed dynamic behaviour of DNA fragments to be studied taking into account water and counterions. The results show that the intrinsic properties of target sequences can play a major role making the duplex susceptible to deformations which promote subsequent protein binding.

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D. Flatters, M.A. Young, D.L. Beveridge, R. Lavery J. Biomol. Struct. Dyn. (1997) submitted.

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2-5

CHEMICAL SHIFT CALCULATIONS: A NEW TOOL FOR MACROMOLECULAR STRUCTURE DETERMINATION

A. Dejaegere, F. Sirockin, M. Karplus, D. Sitkoff, D. Case, J-F. Lefèvre.

NMR is an important technique for structure determination of proteins and nucleic acids. The large number of NMR structures now available has made possible the development of semi-empirical theories for chemical shift dispersion that allow the calculations of chemical shifts in proteins. We used a semi-empirical model to calculate the chemical shifts for PMP-D2 and ω-conotoxin MVIIA; both are small proteins (35 and 25 residues, respectively) with three disulfide bridges. Some regions of the proteins showed large discrepancies between calculated and experimental shifts. Heteronuclear relaxation studies subsequently showed that these discrepancies are due to slow conformational exchange in the molecule. These results showed that chemical shift calculations can serve as a useful independent test of the quality of the NMR structures.

Nucleic acid structure determination by NMR often suffers from a scarcity of experimental NOE restraints; chemical shift information should help in structure refinement. We are developing a model for nucleic acids that combines semi-empirical and density functional calculations of chemical shifts. The model shows that chemical shifts are sensitive to details of nucleic acids structure.

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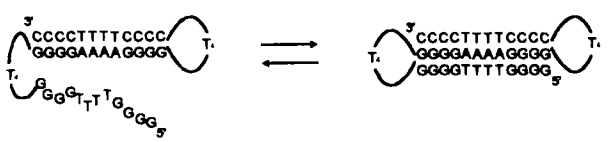
2-6

SPECTROSCOPIC INVESTIGATION OF AN INTRAMOLECULAR TRIPLEX FORMATION WITH AN OLIGONUCLEOTIDE CONTAINING PUR•PUR:PYR AND PYR•PUR:PYR TRIADS AND ITS COMPLEXATION WITH NETROPSIN.

C. Gondeau, M. Durand and J.C. Maurizot

Triple helix formation was investigated with the oligonucleotide d($G_4T_4G_4.T_4.G_4A_4G_4.T_4.C_4T_4C_4$) using UV thermal denaturation analysis and circular dichroism spectroscopy. We demonstrated that in 10 mM Na cacodylate, 0.2 mM EDTA, pH 7.0, the oligonucleotide is in a double stranded structure with a dangling d($G_4T_4G_4.T_4$.) extremity. The dangling extremity does not adopt the tetraplex structure usually observed in these buffer conditions. The presence of $MgCl_2$ promotes the triplex formation, as shown by the CD spectrum which exhibits a negative band at 278 nm characteristic of G•G:C triads.



Thermal denaturation studies shows that the triplex-to-duplex and the duplex-to-coil transitions occur at the same temperature. The observed concentration independence of the melting profiles confirms the intramolecular nature of the structure and rules out the possible existence of intermolecular complexes.

The interaction of netropsin, a DNA minor groove binding drug, with the oligonucleotide was studied. We show that netropsin binds to triplex in a 1:1 stoichiometry, without deplacing the major-groove bound third strand. That binding stabilizes the triplex.

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2-7

STABILITY AND DYNAMICS OF A PNA/DNA COMPLEX

A. Gräslund

PNA (Peptide Nucleic Acid) is a synthetic DNA analog in which the bases are the same as in DNA, but the backbone is composed of a pseudo-peptide chain without negative charges. PNA hybridizes with DNA of complementary base sequence and the hybrid complex is more stable towards thermal denaturation than the corresponding DNA/DNA duplex. PNA has a potential for pharmaceutical and biotechnological applications, but is also useful for determining how charge interactions influence DNA structure and dynamics.

We have used NMR spectroscopy to determine that a PNA/DNA hybrid duplex has its DNA strand in a B-like conformation (1). NMR has also been used to determine the base pair opening kinetics (on the ms time scale) of the hybrid duplex. The results show that the kinetics are significantly changed in the PNA/DNA duplex compared to a corresponding DNA/DNA duplex, with the PNA bases generally having significantly increased opening and closing rates (2). The stability towards thermal denaturation under conditions of varying ionic strength was studied for a PNA/DNA duplex. The results could be interpreted using Poisson-Boltzmann and counterion condensation theories (3).

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2-8

MOLECULAR DYNAMICS INVESTIGATIONS OF THE HAMMERHEAD RIBOZYME RNA.

T. Hermann[1], Pascal Auffinger[1], William G. Scott[2], and E. Westhof[1].

The hammerhead ribozyme is a catalytically active RNA that cleaves its own backbone. Crystal structures of hammerhead RNA are available, revealing the presence of several Mg^{2+} ions probably involved in the catalysis of the ribozyme. However, the static crystal structures do not allow to decide which of the mechanisms postulated for ribozyme self-cleavage is correct.

In order to study the dynamical behaviour of the ribozyme, we performed molecular dynamics simulations on fully hydrated and neutralized hammerhead RNA in presence of the Mg^{2+} ions located at the sites determined in the crystal structure analyses.

The results of our simulations provide evidence for a μ-bridging OH^- ion between two Mg^{2+} ions that are close to the cleavable phosphate in the crystal structure of the ribozyme. It was proposed earlier that during self-cleavage of the ribozyme a hydroxide bound to a divalent cation abstracts the proton from the cleavage-site 2'-hydroxyl, which then attacks the adjacent phosphate. We suggest that the μ-hydroxo-bridged cluster is superior to a single Mg^{2+} in stabilizing a high local concentration of OH^- in proximity of the hammerhead ribozyme's active site, and the activity of OH^- ions around the metal cluster may thus be higher.

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2-9

SELECTIVELY 13C-ENRICHED DNA : EVIDENCE OF FAST AND SLOW INTERNAL MOTIONS. F. Gaudin, F. Paquet, N.T.Thuong, D. Genest and G. Lancelot.

Relaxation rate measurements can be used to provide a detailed description of nucleic acids dynamics. These data depend on the global and internal motions of the macromolecule, as well as the distance between the nucleus under investigation and its surrounding.

The use of ^{13}C-NMR relaxation parameters is attractive because generaly the relaxation process of the ^{13}C are governed by the bonded protons whom the C-H distance is constant. Thus, the interpretation of the relaxation parameters is reduced to the analysis of the motions of the ^{13}C-^{1}H vectors. In order to increase the low natural abundance of the isotope, selectively ^{13}C-labeled molecules was required.

Using ^{13}C-selectively enriched oligonucleotides on the C1' position, we have shown that :

- the sugar exhibited a restricted rotation around the glycosidic bond by ±28° with an internal diffusion coefficient of $30x10^9$ rad^2 s^{-1}. Analysis of the distributions and of the autocorrelation functions of the glycosidic angle fluctuations χ, using a 250ps trajectory of d(CGCAAATTTGCG)$_2$ gave data in agreement with the analysis of the experimental results $\Delta\chi$=±20° and D_f=$17x10^9$ rad^2 s^{-1}.

-direct evidence for the presence of a conformational exchange process has been obtained for the residue A4 from rotating frame relaxation measurements. This additional relaxation process involves large amplitude and slow motions (τ_{ex} = 130µs) which can reflect special dynamic of the tract AAATTT which support the spin of hydration. Such an exchange process was also observed on residues of the lac operator (double helix « isolated » in solution) whiuch are in close contact with the protein in the complex with the *lac*-repressor recognition process.

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2-10

DYNAMICS OF THE GENOME - FROM SUPERHELICAL DNA TO CHROMOSOMES.

Jörg Langowski

At many levels of organization one may view DNA and chromatin as flexible polymers and approximate them through chains of rigid segments that encompass tens to thousands of base pairs. Their interactions are determined by global properties of the chain and its environment: bending and twisting rigidity, electrostatic charge of the polymer and ionic strength of the medium, and hydrodynamic interactions between different parts of the chain. Monte-Carlo and Brownian dynamics methods applied to such chain models of superhelical DNA and oligonucleosomes successfully describe experimentally accessible quantities, such as diffusion and sedimentation coefficients, and dynamic light scattering data. Recent small angle neutron scattering experiments showed the existence of intramolecular interference between opposing strands of the superhelix at low ionic strength, in good agreement with our model predictions. The models also allow to make detailed predictions about structural rearrangements in DNA, e.g. induced by local bending.

The organization and dynamics of the interphase chromatin fiber can again be described by a polymer chain. Similar to metaphase, the interphase chromatin fiber is folded into 100 kb loops, and its structure and dynamics are determined by Brownian motion. Predicted interphase distances of genetic markers in the 10 to 100 Mbp range are in quantitative agreement with FISH measurements of distances between genetic markers. Size, shape and flexibility of chromosomes are described as well as the positions of genes relative to the chromosome territory. From the simulated structures we generated 'virtual' multi-color images of FISH experiments, which allow us to quantify the structure of chromosome territory boundaries. Also, differential painting of chromosome arms or R- and G-bands is simulated, and the computed images are compared with experimental confocal section series.

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2-11

SOLUTION STUDY OF DNA POLYMERASE INTERAC-TION WITH DNA TEMPLATE.PRIMERS AND DEOXYNUCLE-OSIDE-5'-TRI-PHOSPHATES. O.I. Lavrik

The interaction of synthetic DNA template.primers and dNTPs with DNA polymerases from eukaryotes, prokaryotes and viruses was analyzed by using method of affinity labeling and kinetic measurements Kd and Gibbs energy values for template, primer and dNTP complex formation with DNA polymerases have been evaluated from their competition with reactive DNA and dNTP derivatives. The general model of DNA template.primer and dNTP interaction with DNA polymerases in solution was suggested on the basis of the data (Kolocheva et al. 1991 ; Lavrik 1991). Photoreactive base-substituted dNTP analogs were synthesized and used as a tool to study of dNTP interaction with DNA polymerases (Wlassoff et al. 1995). Two ways of specific modification of DNA polymerases were applied. In one approach dNTP analogs was introduced into the primer by DNA polymerases before UV-irradiation. In an alternative approach dNTP analogs were first UV-crosslinked to enzyme. Subsequently radiolabeled template.primers were added to analyze the substrate activity of crosslinked dNTP in template dependent and nontemplate nucleotide addition catalyzed with DNA polymerases under different conditions. The data permit to describe the influence of template on dynamics of dNTP interaction with active sites of mammalian DNA polymerase α, DNA polymerase β, Klenow fragment DNA polymerase I and human immunodeficiency virus reverse transcriptase (Lavrik et al. 1996).

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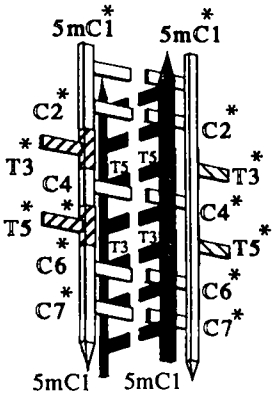
2-12

BI-STABLE DNA I-MOTIF SWITCHES AND CONVERSION KINETICS. J-L Leroy and S. Nonin.

Tetramers formed by C-rich oligomers at low pH are composed of two parallel-stranded duplexes whose hemi-protonated C·C$^+$ pairs intercalate in a head-to-tail orientation in a so-called i-motif. In all cases reported previously the duplexes are identical.

The tetramer formed by the d(5mCCTCC) oligomer is different. The structure computed on the basis of 55 inter-residue distances derived from NOESY experiments shows two non-equivalent duplexes whose intercalation order is C5* 5mC1 C4* C2 (T3*) T3 C2* C4 5mC1* C5. In one duplex the thymidines T3 stack as a T·T pair but the thymidines (T3*) of the other duplex are turned outwards in the wide grooves of the tetramer. Each duplex is symmetrical.

Numerous exchange cross-peaks provide evidence for duplex interconversion, with opening of the T3·T3 pair and closing of the T3*·T3* pair and vice-versa. The interconversion rate is 1.4 s^{-1} at 0°C.



Opening of the C4·C4$^+$ and C4*·C4*$^+$ pairs, and dissociation of the tetramer are not part of the interconversion since they occur at much slower rates. d(5mCCUCC) adopts a similar structure, but the rate of duplex interconversion is faster (40 s^{-1} at 0°C).

The tetramer of d(5mCCTCTCC) exhibits a similar behaviour (scheme). In one duplex the thymidines form two symmetrical T·T pairs (T3·T3 and T5·T5) while the thymidines T3* and T5* of the second duplex are turned outwards. In other words, the states of the two switches are fully inter-dependent.

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2-13

FLUORESCENCE POLARIZATION ANISOTROPY OF A TRIPLE HELIX SYNTHETIC DNA OLIGOMER. F. Barone, G. Chirico, M. Matzeu, F. Mazzei, F. Pedone.

A 27 bp homopurinic-homopyrimidinic duplex DNA was annealed with a complementary third strand 21 bases long. The formation of the triple helix was checked both by circular dichroism spectra and gel electrophoresis measurements. The triplex to duplex form transition, as monitored by UV absorption measurements, occurs from 25 to 45°C. We have followed this transition by fluorescence polarization anisotropy (FPA) measurements of ethidium bromide intercalated in the triplex oligomer from 10 to 40°C and compared with similar measurements for the duplex form. By FPA measurements of the EB bound to DNA fragments the usual hydrodynamic parameters can be derived. At low temperature the hydrodynamic radius R_H of the triple helix is 12.6 ±0.3 Å and that of the duplex is 11.2 ±0.3Å. Starting from 25°C the R_H values of the triplex diminishes to near the value of the duplex, showing that the triplex to duplex transition can be monitored by FPA measurements.

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2-14

CHANGE OF DNA LENGTH AS A RESULT OF DNA BINDING PROTEIN INTERACTION : MEASUREMENT BY OPTICAL TWEEZERS MANIPULATION K. Sakata-Sogawa, M. Kurachi and H. Tashiro

DNA looping is generated by direct interaction between two DNA binding proteins located at separated positions on a DNA molecule. It is expected to play an important role in transcription reguration. We have developed a system measuring molecular length changes of single DNA molecules aiming at direct observation of DNA looping formation. Each end of a single DNA molecule with known base pair numbers was attached to polystyrene beads. Both beads were trapped and manipulated independently with optical tweezers. A trapped DNA molecule was then stretched and the molecular length was estimated from the distance between the two beads. The molecular length was found to be proportional to the base pair number. The rise per residue was evaluated to be 3.4 Å. The length measurement was applied to DNA fragments containing GC box sequences at two different locations separated by a distance of 2.3kbp. The addition of GC box binding transcription factor shortened the molecular length. This result suggested the formation of DNA looping by the interacion between transcription factors.

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2-15

DNA PHOTOPHYSICS: DUAL FLUORESCENCE FROM ADENINE DERIVATIVES. B. Albinsson.

The nucleic acid bases are non-fluorescent at room temperature due to a very efficient radiationless deactivation of, presumably, their lowest excited singlet states. In aqueous solution at room temperature, adenosine has a fluorescence quantum yield of 6×10^{-5} [1] and the lifetime of the lowest singlet excited state is about 1 ps [2]. Since no efficient irreversible photochemical transformation of the adenine chromophore has been observed it has been suggested that the deactivation is purely photophysical, but the nature of this process is still unknown. We have decided to systematically investigate different purine and pyrimidine derivatives with the aim of characterizing their photophysical properties as a function of e.g. substitution pattern, solvent polarity and temperature. Here we report on the novel observation that the adenine derivative N^6,N^6-dimethyladenosine (DMA) shows dual fluorescence; in addition to the "normal" adenine fluorescence at 330 nm, this derivative shows a red-shifted emission around 500 nm. The long wavelength emission is suggested to originate from a conformationally distorted species formed in an intramolecular excited state reaction. Radiationless deactivation of DMA occurs almost exclusively through isomerization and *not* by direct internal conversion and the ground state recovery time at room temperature is expected to be in the ns rather than in the ps range.

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[1] Cadet, J.; Vigny, P. in *Bioorganic Photochemistry*, Vol. 1, Morrison, H. Ed.; John Wiley & Sons: New York, 1990; 1-272.
[2] Indirect estimation: J. Eisinger and A. A. Lamola, in *Excited States of Proteins and Nucleic Acids* (R. F. Steiner and I. Weinryb, eds.), Plenum Press, New York, (1971); Direct measurement: D. N. Nikogosyan, D. Angelov, B. Soep and L. Lindqvist, *Chem. Phys. Lett.*, **252** (1996), 332.

2-16

ONE-ELECTRON OXIDATION OF DNA: SEQUENCE EFFECT. D. Angelov , B. Beylot and A. Spassky.

In the present work, we were interested in the influence of DNA structure on the one-electron oxidation products of guanine. We have directly photoionized DNA sequences by exposure to high intensity UV (266nm) laser nanosecondes pulses. Homo or hetero-duplexes, including guanines in various environments as well as Gn runs were used as templates. Two different types of lesions, 8-oxodG and oxazolone resulting respectively from the hydration and the deprotonation of the guanyl radical have been pointed out and quantified, at the nucleotide level, by using enzymatic (Fpg protein) and chemical attack. Great variations were observed in the sum as well as in the relative yield of each type of lesion at individual guanine of the DNA sequences. Results are discussed in terms of a relationship between intramolecular rearrangements of the guanyl radical cation and DNA helical conformation and dynamics, depending on the sequential context.

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2-17

RENATURATION OF CONDENSED DNA. I. Chaperon , J.-L. Sikorav.

DNA renaturation has been extensively studied and provides a most useful tool for molecular biology (1). In the presence of monovalent cations the rate of the reaction is controlled by an energy of activation. It is possible to increase this rate by 10^2-10^3 fold in the presence of multivalent cations, leading to rates compatible with a diffusion-controlled mechanism (2). It has been proposed that this latter reaction involves as a transient step the condensation of the (denatured) single-stranded DNA molecules.

In order to clarify this mechanism, we have studied the condensation by multivalent cations of single stranded DNA molecules in the absence of their complementary strands. We have obtained phase diagrams for the aggregation of these molecules in the presence of monovalent and multivalent cations. The aggregation process was monitored by centrifugation of ^{32}P labelled DNA molecules and determination of the amount of precipitated DNA. Renaturation rates have been determined at different locations of the phase diagrams by gel electrophoresis. Our results show the existence of a strict correlation between the condensation of single stranded DNA molecules and the acceleration of the renaturation rates.

(1) J. G. Wetmur. 1991. Crit. Rev. Biochem. Mol. Biol. 26(3/4):227-259.
(2) J.-L. Sikorav and G. M. Church. 1991. J. Mol. Biol. 222:1085-1108.

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2-18

SEQUENCE SPECIFIC DNA CLEAVAGE MEDIATED BY PHOTOEXCITED PAZELLIPTINE : INVESTIGATION OF THE MECHANISM BY TIME RESOLVED ABSORPTION AND FLUORESCENCE SPECTROSCOPY. E. Renault*, C. Videlot*, F. Tfibel*, M.P. Fontaine-Aupart*, M. Charlier$, R. Pansu

Pazelliptine (PZE), a 9-azaellipticine derivative, is known to be a potent antitumor agent. The biological effects of this compound arise from its interaction with DNA and metabolic activation. Furthermore, we have shown that upon irradiation (λ > 350 nm) PZE may photosensitize the formation of single (ssb) and double (dsb) strand breaks in double stranded supercoiled DNA. A linear relationship between ssb and the fluence is observed under aerobic conditions. The nucleotide sequence analysis revealed that the strand cleavage reaction occurs preferentially at the G base site. Thus, PZE appears as a new suitable probe for DNA recognition and a tool for biochemical application, biotechnology and photodynamic therapy. Dynamic investigations of photophysical and photochemical processes can provide a wealth of information on factors which preclude the photodynamic action of the drug. We have developed a study of the singlet and triplet excited states of PZE intercalated in different polynucleotides and DNA by time resolved (picosecond and nanosecond) fluorescence and absorption measurements. The fluorescence of PZE bound to poly(dG-dC)-poly(dG-dC) appears to be quenched. This non radiative transition $S_1 \rightarrow S_0$ is due to an efficient charge transfer between the excited drug and the nucleotide bases. The study of the triplet state of PZE reveals an electron transfer between this excited state and the A-T base pair leading to the formation of the radical cation of the nucleic acid identified by its absorption spectrum. Thus, the formation of the singlet and/or triplet excited species may account for the toxic effects of the drug under the influence of far UV light.

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2-19

INTERSTRAND CROSS-LINKING REACTION IN TRIPLEXES CONTAINING A MONOFUNCTIONAL TRANSPLATIN-ADDUCT. C. Colombier, B. Lippert and M. Leng.

Our purpose was to determine whether a single transplatin monofunctional adduct, either *trans*-$[Pt(NH_3)_2(dC)Cl]^+$ or *trans*-$[Pt(NH_3)_2(dG)Cl]^+$ within a homopyrimidine oligonucleotide, could further react and form an interstrand cross-link once the platinated oligonucleotide was bound to the complementary duplex. The single monofunctional adduct was located at either the 5'-end or in the middle of the platinated oligonucleotide. In all the triplexes, specific interstrand cross-links were formed between the platinated Hoogsteen strand and the complementary purine-rich strand. No interstrand cross-links were detected between the platinated oligonucleotides and non-complementary DNA. The yield and the rate of the cross-linking reaction depend upon the nature and location of the monofunctional adducts. A major point was that the half-lives of the monofunctional adducts within the triplexes were in the range 2-6 h. The potential use of the platinated oligonucleotides to modulate gene expression is discussed.

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2-20

RECOGNITION OF A C-RICH TELOMERIC OLIGONUCLEOTIDE BY A PROTEIN FROM YEAST. J-F. Cornuel, A. Moraillon, M. Leblond and M. Guéron

The i-motif, a new DNA structure, is built by the intercalation of two duplexes, each of which includes parallel strands with hemi-protonated C·C+ pairs. Considering that this structure may be significant for centromeres and telomeres, we have searched for proteins which can bind to the yeast telomeric fragment:
d((CCCACA)3CCC) .
We have observed by an electrophoretic retardation assay, the association of at least one protein with this sequence. From gel filtration experiments the molecular weight of the protein is around 120 kDa . SDS-PAGE suggests that the protein is a multimer. Direct comparison with RAP1 and with extracts from SIR-deficient yeasts (kindly provided by E. Gilson, Ecole Normale Supérieure de Lyon) shows that the protein is not RAP1, SIR1, SIR3 or SIR4.

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2-21

CRYSTALLIZATION OF A DNA DODECAMER CONTAINING AN INTERSTRAND *TRANS*-DIAMMINE-DICHLOROPLATINUM(II) ADDUCT. F. Coste, J.M. Malinge, N. Hervouet, M. Leng and Ch. Zelwer.

The reaction between transplatin and an oligonucleotide containing a GC base pair leads to the formation of an interstrand cross-link (ICL) between the G and the complementary C residues. A biophysical study reveals that this adduct bends the double helix by ≈ 26° and unwinds it by ≈ 12°. It has been shown that the values of distortion angles induced in DNA by platinum complexes are important structural parameters for its recognition by specific DNA-binding proteins.
A 12-mer double-stranded oligonucleotide of sequence d(CTCTCG*AGTCTC).d(GAGACTC*GAGAG) with an ICL between the G6 N-7 and the C18 N-3 positions was prepared and purified. Crystals of this modified duplex have been obtained by the vapor diffusion method with MPD as a precipitant in cacodylate buffer (pH 6.0 to 7.0). Crystallizations occurs in various conditions according to the pH, the relative ratio [$MgCl_2$]/[spermine], the temperature and the ionic strength of the solutions. Crystals with maximum dimensions 0.03 x 0.03 x 0.1 mm have been tested on a MarResearch imaging plate system using the Cu $K\alpha$ radiation. Preliminary X-ray diffraction images have been collected.

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2-22

PHOTOPHYSICS OF ATP, II. S. Fu., L. P. Hart and M. Daniels.

Earlier time-domain investigations of the adenyl chromophore in aqueous solution at room temperature have revealed unexpected complexity. Lifetimes of 290 ps and 4.17 ns have been reported for ATP excited at 290 nm, 5 ps and 330 ps for 9-MA excited at 285 nm, and 1.6 ps and 2.1 ps for Ado and AMP (2-photon excitation at 280 nm). Singular value decomposition (SVD) of spectral data matrices for ATP excited at 290 nm indicate the presence of three independent emitters. The present work uses deconvolution of time-delayed (Δt = 0-2 ns) time-windowed (δt = 100 ps) spectra in conjunction with multi-wavelength lifetime resolution at concentrations from 0.2-20. mM to investigate this behavior. Excited at 290 nm, decomp are multi-component at 340 nm, 360 nm and 420 nm. Characteristically two major components differing by an order of magnitude in lifetime (τ_1 ~150-300 ps, τ_2 ~2-4 ns) are obtained. When a third component results, its lifetime is an order of magnitude greater again but it accounts for <1% of total decay. Time-windowed spectra have been resolved using lifetimes of 0.2 ns and 3.3 ns. The spectrum corresponding to the ns lifetime presents a smooth unimodal curve, λ_{max} 405 nm which, ratioed to the internal resonant raman peak, is essentially concentration independent from 2mM to 20mM. The ps spectrum behaves quite differently: its profile changes with concentration as does its intensity, indicating that it does not originate from a single species. This spectrum has been self-consistently resolved into two components, one with λ_{max} at 362 nm, scaling lineally with concentration, the other with λ_{max} at 398 nm and scaling with [concentration]^2. Other lifetime and pH studies have been carried out.

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2-23

THE B-Z DNA TRANSITION : A POISSON-BOLTZMANN INTERPRETATION OF THE EFFECTS OF DI- AND TRI-VALENT CATIONS. J.-Ph. Demaret and M. Guéron.

Over the past years, we have developed simple-minded models of polyelectrolytes to show that the association of ions to nucleic acids is mostly electrostatic. Hence specific coordination is either absent or provides very little free energy, and is not an important determinant of nucleic acid structure.

An illustration is the B-Z transition of d(GC).d(GC) DNA which may be driven by increasing the ion concentration (Na or Mg). The free energy measurements in the Na case may be compared directly to electrostatic theories. The electrostatics is sufficiently described by the Poisson-Boltzmann theory; B and Z-DNA are represented by porous cylinders which reflect their main structural difference: in B-DNA, the charged phosphate groups are much better immersed in the solution and hence better screened.

But in some cases, ions have an effect so strong that it is often considered to involve non-electrostatic factors: for instance, in d(5-methylCG). d(5-methylCG), the B-Z transition is induced by Mg(2+) at mM concentrations or less, whereas the required Na concentration is 1.1 M. Nevertheless we show that both situations fall within the scope of the simple electrostatic model. The model also accounts for the driving of the B-Z transition by cobalt hexamine(3+) at submicromolar concentrations. In this case, association of the complex to DNA must be taken into account, but the equilibrium constant required to fit the data corresponds to a rather weak affinity, in agreement with the general picture.

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2-24

Hammerhead ribozymes: structure-activity relationships *in vitro* and *ex vivo*. A.Favre°, C.Saintomé°, P.Laugâa°, J.-L. Fourrey*

The folding in solution of a bipartite hammerhead ribozyme domain assembled from a 34-mer ribozyme and 14-mer deoxy photoactivable substrate analogues has been explored using either cleavage conditions (20 mM Mg^{2+}) or the presence of 400 mM Na^{+}. The pattern of crosslinks obtained is essentially the same under both conditions and allowed elaboration of a plausible structure by molecular modeling. The overall Y conformation with stems II and III roughly coaligned and stems I and II side by side is in agreement with other solution data and retains most of the features observed in crystals. Our data indicate however a high flexibility of the catalytic core and that the loop II (A2-6) and top of stem I (distant from 24 A in the crystal) can come into contact. The corresponding crosslink retains full cleavage activity however and construction of alternative ribozyme forms has been undertaken to constraint the catalytic core. Transcripts of 192 nt and 134 nt carrying this 34 nt ribozyme expressed in Hela cells were found active against a 692 nt RNA target derived from VIH but inactive *in vitro* even when faced to the small 14-mer substrate whatever the conditions used, thus suggesting that the transcripts conformations are distinct *in vitro* and *ex vivo* and/or that cellular factors (proteins) favors formation of catalytically active complexes.

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2-25

KINETIC STUDY OF A (T,C)-MOTIF TRIPLE HELIX BY BIOMOLECULAR INTERACTION ANALYSIS TECHNOLOGY : A COMPARATIVE STUDY OF SOLUTION AND BIOSENSOR-BASED METHODS. Jian-sheng Sun, Thérèse Garestier & Claude Hélène

Over the past ten years, intermolecular triple helix formation has aroused a great deal of intense research due to potential applications in molecular biology and therapeutics. A number of studies have been aimed at characterizing the thermal stability as well as the thermodynamics of triple helix formation. However, kinetic data on triple helix formation are limited and fragmentary.

Biosensor technology allows studies of molecular interactions in realtime. It provides a direct access to kinetic data on triple helix formation. The present work compares the kinetic parameters of triple-helix formation obtained using the BIA technology with those previously determined by analysing the hysteresis of melting curves obtained by heating and cooling triplex solutions using UV spectrophotometry (Rougée *et al.*, *Biochemistry*, **1992**, 31, 9269-9278). Several factors affect the kinetic parameters measured by the biosensor technology :

1) the presence of dextran molecules on the sensor chip ;
2) the stability of baseline after a regeneration pulse ;
3) the length of the linker between the duplex and the biotin ligand used to immobilize the duplex on the streptavidin-substituted dextran ;
4) the ratio of biotin to streptavidin and the density of surface-bound streptavidin ;
5) the time allowed for the dissociation of the triplex ;
6) the temperature range of measurement.

All these factors have to be taken into account to determine meaningful kinetic parameters.

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2-26

THE MOLECULAR MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY THE cAMP RECEPTOR PROTEIN OF *ESCHERICHIA COLI*. P. Eichenberger, S. Déthiollaz, M. Engelhorn and J. Geiselmann.

The cAMP receptor protein (CRP) activates transcription by binding to sites upstream of the target promoter. Direct protein-protein contacts with the alpha subunit of RNA polymerase are responsible for activation when CRP is bound immediately upstream of the promoter (around position -60). However, transcriptional activation persists when the CRP binding site is moved further upstream by increments of complete turns of the DNA helix. Activation from these far-upstream positions is mostly due to interactions of the DNA upstream of the CRP binding site with the back-side of RNA polymerase. CRP favors such interactions by bending the DNA.

We use UV-laser footprinting to detect structural transitions within different activation complexes during the process of transcriptional initiation and activation. These kinetic structural data show that CRP stabilizes the open complex at the *malT* promoter without affecting the rate of its formation. The interactions of the far-upstream DNA with RNA polymerase are established late during initiation and serve to favor these open forms of the promoter. These interactions thereby increase the rate of promoter escape. The physiological relevance of the mechanism is confirmed by *in vivo* UV-laser footprints.

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2-27

Ca^{2+}, Mn^{2+} AND Cu^{2+} IONS INTERACTION WITH DNA AND POLYPHOSPHATES IN SOLUTIONS WITH DIFFERENT WATER ACTIVITY. E.Hackl, S.Kornilova and Yu. Blagoi.

The DNA and polyphosphates complexes with Ca^{2+}, Mn^{2+} and Cu^{2+} ions in water and water-alcohol solutions at different metal contents were studied by IR-spectroscopy. All the investigated ions induce the frequency shifts in absorption bands of DNA phosphate groups and bases. Dependences between intensities of DNA absorption bands and metal ion concentration were obtained in the wide ion concentration interval. The same dependences were obtained for mixed solutions containing different (5, 9, 13% v/v) ethanol, glycerol or propandiol concentrations. We suppose that the metal ion binding to DNA different groups induce the compactization of the DNA macromolecule. That process has a cooperative character. Binding isotherms are of nonmonotonous character similar to Van-der- Vaals isotherms for liquid-vapour phase transitions. The sites of metal ions binding to DNA were determined. The results obtained permit us to propose the model of divalent metal ions binding to DNA and to estimate the binding constants and parameters of cooperativity of the ion binding to phosphate groups and bases of DNA. Mixed solutions change the cooperative parameter and binding constant. It can be seen that the metal ion binding to DNA depends extremely on the water activity.

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2-28

NMR STRUCTURE OF AN INTRAMOLECULAR I-MOTIF. X. Han, M. Guéron and J.L Leroy

The i-motif, a DNA structure of intercalated $C{\cdot}C^+$ pairs, may be significant for centromeres and telomeres. We now report the solution structure of the i-motif formed by the intramolecular folding of $d(5mCCT_3C_2T_3AC_2T_3C_2)$.

Gel filtration chromatography shows that the structure formed by this oligonucleotide is monomeric. There are four $C{\cdot}C^+$ pairs as shown by the NMR spectrum which includes four cytidine imino proton peak, and each with NOE cross peaks to amino protons of two cytidines. They are intercalated into an i-motif which forms the core of the structure. The TTT segment loops across the two narrow grooves, while the TTTA segment loop across one of the wide grooves. The i-motif core is extended by the T5·T16 base-pair (lifetime 6 ms, at 0°C) which stacks on the $C2{\cdot}C13^+$ pair. In the T8T9T10A11 loop, T8 H3 is H-bonded to A11 N7. At 0°C, the base-pair lifetimes are in the range of seconds for the inner $C{\cdot}C^+$ pairs ($5mC1{\cdot}C12^+$ & $C2{\cdot}C13^+$), and of milliseconds for the outer pairs ($C7{\cdot}C18^+$ & $C2{\cdot}C13^+$). The structure is half unfolded at pH 7.4, 0°C. As the pH raised, the dissociation of the four $C{\cdot}C^+$ pairs is not fully cooperative.

The $d(5mCCTA_2C_2T_3AC_2TA_2C_2)$ and $d(5mCCAT_2C_2T_3AC_2AT_2C_2)$ sequences are closely related to the Bombyx Mori telomere and of the human centromere whose repeat units are, respectively, (C_2TA_2) and (C_2AT_2). They form intramolecular i-motif with the same intercalation topology as the sequence described above. Both are stable at pH 7, 0°C. The study of many analogous oligonucleotides indicates that the ability to form intra- or inter- loop A·T or T·T pairs may effect the stability of the intramolecular i-motif..

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2-29

A COMPARATIVE STUDY OF DNA INTERACTION WITH MONO- DI- AND TRIVALENT METAL IONS AND COORDINATION COMPOUNDS OF Pt(II) AND Co(III). N. A. Kasyanenko, E. F. Haya Enriquez , L. V. Plotnikova, A. Zanina, and E. V. Frisman

The optical anisotropy, intrinsic viscosity and spectral characteristics of DNA in solution have been studied by the methods of flow birefringence, viscometry, circular dichroism and UV - spectroscopy. It was shown that the binding of divalent ions to DNA leads to the changing in its polyelectrolytic swelling as compared with the screening effect of monovalent ions. Note that the decrease of the size of macromolecule is not very sensitive to the nature of divalent cations and their binding positions. At high concentration of mono- and divalent ions the difference in their influence on the size of DNA molecule disappears. The optical anisotropy measurements have shown that in these conditions the influence of great quantity of counterions on the orientation of hydration water of DNA takes place. The binding of the trivalent iron ions to DNA leads to its compaction. The compaction is accompanied by the increasing in the DNA optical anisotropy. It is suggested that the formation of compact structure is under way during the iron ions binding to DNA phosphate groups distant along the chain. In this case the appearing of mutually oriented DNA parts ensure the increase in the optical anisotropy of DNA. The persistence length of DNA does not change during the di- and trivalent metal ions binding at ionic strength $I > 0.002$ *M*. The DNA interaction with some coordination compounds of Pt (II) and Co(III) was studied. The results are in agreement with those obtained previously.

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2-30

DISCOTIC LIQUID CRYSTALLINE PHASES OF NUCLEOSOME CORE PARTICLES. A. Leforestier and F. Livolant

Although the first level of DNA folding into the nucleosomic fiber and the structure of the nucleosome itself are well established and documented, higher orders of Eucaryotic chromatin organization and folding remain poorly understood. Using in parallel polarized light microscopy, freeze-fracture electron microscopy and cryoelectron microscopy of vitrified thin films, we investigate the supramolecular organization of condensed phases of nucleosome core particles obtained either in highly concentrated solutions (in presence of monovalent salt), or by precipitation with polycations (spermidine 3^+). In both cases, nucleosome core particles organize into discotic columnar hexagonal liquid crystalline phases which concentrations can be compared to those found in a cell nucleus, ranging from about 250 to 500 mg/ml. In these phases, the nucleosome cores stack the ones on top of the others into columns that form a 2-dimensional hexagonal lattice. In the first case, the columnar hexagonal phase is governed by net repulsive excluded volume interactions (good solvent conditions), while in presence of polycations, the liquid crystalline organization is obtained *via* net attractive interactions (poor solvent).

One of the main differences between this model system and the nucleosomic fiber of eucaryotic chromatin is the lack of ADN linker. In order to take into account this parameter, we now intend to investigate the organization of condensed phases formed by di-, tri- and poly-nucleosomes.

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2-31

STABILITY AND DYNAMICS OF HYDRATED D N A IN THE DEPENDENCE ON NUCLEOTIDE COMPOSITION. *V.Maleev, M.Semenov and A.Gasan.*

Infrared spectroscopy and calorimetry have been used to investigate the process of formation of the DNA structure and its hydration environment depending upon nucleotide composition. The AT- and GC-rich DNA and also double helix polynucleotides AT(AU) and GC-type in hydrated samples with variable water content have been studied. From the analysis of infrared parameters (frequencies and intensities of several main bands) in the dependence on the number of water molecules per nucleotide (n) three stages of hydration have been found in which three structural states (unordered, A- and B-form) of DNA are realized The distribution of the water molecules over the binding energy with DNA (redunant hydration energy $\Delta E(n)$) have been determined using the direct microcalorimetry measurements of the water evaporation from hydrated DNA samples and also the IR spectroscopic data in water sorbed on DNA. The dynamic properties of the DNA-water system were characterized by the dipole relaxation time and by the free volume of the water molecules in the hydrate environs of DNA. These parameters have been determined using the values of the redundant hydration energy $\Delta E(n)$ for DNA with different nucleotide composition. The most important result of the present study is the marked difference between values of $\Delta E(n)$ for DNA of AT- and GC-type. The obtained results are discussed with respect to the hydration nature of the difference between stabilities and dynamical properties of AT- and GC-pairs in DNA.

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2-32

REARRANGEMENT OF INTERSTRAND CROSS-LINKS INTO INTRASTRAND CROSS-LINKS IN *CIS*-DIAMMINEDICHLOROPLATINUM(II)-MODIFIED DNA.

J.-M. Malinge, C. Pérez and M. Leng.

Intrastrand and interstrand cross-links are mainly formed in the reaction of the anticancer drug *cis*-diamminedichloroplatinum (II) (*cis*-DDP) with DNA. It is accepted that these biologically important lesions are kinetically inert. We have shown that at 37°C interstrand cross-links (ICL) are labile. The ICL instability was first studied within a 10 base pairs (bp) double-stranded oligonucleotide containing a single site specific ICL resulting from chelation of the N7 position of two guanine residues on the opposite strands of DNA at the d(GC/GC) site by a *cis*-diammineplatinum(II) residue. As investigated by electrophoresis under denaturating conditions, the bonds between the platinum and the N7 of guanine residues within the interstrand adduct are cleaved. This cleavage leads to the formation of monofunctional adducts which subsequently form intrastrand cross-links. In 50 mM NaCl, the cleavage reaction is irreversible allowing its rate measurement ($t_{1/2}$=29 h). Within a longer cross-linked oligonucleotide (20 bp), ICL are apparently more stable as a consequence of monofunctional adducts closure back to ICL. We conclude that the ICL cleavage is reversible in DNA and that these adducts rearrange finally into intrastrand cross-links.

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CHARACTERIZATION AND STABILITY OF Py Pu Py TRIPLEX OLIGONUCLEOTIDES FORMED ON NICKED DUPLEXES.

F.Barone, M.Matzeu, F.Mazzei, F.Pedone.

One of the specific targets in antigene strategy is to realize triplex structures which are stable under physiological conditions. In this paper we are aimed at characterizing the physico-chemical properties and the stability of triplexes formed by annealing a pyrimidinic oligonucleotide with nicked purine-pyrimidine duplexes. Nicks are introduced in the synthesis phase on the purinic or pyrimidinic strand of two (21 or 27 base pair long) duplexes. We performed our study by means of gel electrophoresis, UV absorbance melting, circular dichroism (CD) and fluorescence spectroscopy techniques. The analysis of the electrophoretic mobility and CD spectra show that triplex structures are also formed on the defective duplexes studied. Moreover, the introduction of nicks produces negligible differences of electrophoretic mobility both in duplexes and triplexes and CD spectra confirm that the overall conformation of the intact molecule is preserved in the defective samples. Fluorescence measurements indicate that ethidium bromide intercalates in the studied triple helix structures and a lower number of binding sites as compared to the duplex is found. UV melting experiments show that, while nicks induce dramatic destabilization in duplex, minor changes are observed in triplex. In particular, the 27 bp triplex with a nick in purinic strand shows a small increase in stability, with unchanged van't Hoff enthalpy difference. A small decrease in stability and more considerable lowering of enthalpy is observed when the pyrimidinic strand is nicked. Nicks in the 21 base pair oligonucleotide (with a different sequence), give an even more stable structure of triplex with respect to duplex forms. These results indicate that stable triplex structures can be also formed on nicked duplex targets.

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2-34

Repliement d'un ADN riche en cytosines et reconnaissance par des protéines: implications pour les télomères.

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Le génome humain comprend de très nombreuses sequences répétées. Certaines de ces séquences, présentes au niveau des centromères et surtout des télomères sont riches en cytosines, et peuvent adopter une conformation particulière, dite "ADN-i", basée sur l'existence de paires de bases C.C+.

Un ADN simple-brin de séquence identique aux télomères des chromosomes humains $(CCCTAA)_n$ est susceptible de former le motif i, tandis qu'un ARN simple brin de séquence correspondante ne forme pas celle-ci à pH neutre. La formation du motif i est optimale à pH acide, mais reste possible à pH physiologique. Cela nous a incité à rechercher des protéines nucléaires qui se fixent sur une telle structure. Il existe dans les cellules HeLa une ou plusieurs protéines abondantes reconnaissant spécifiquement la séquence correspondant au brin riche en cytosines des télomères humains. Nous poursuivons la purification de ces facteurs, grâce au criblage d'une banque d'expression d'ADNc.

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2-35

MODIFICATION OF DNA DUPLEXES TO SMOOTH THEIR THERMAL STABILITY INDEPENDENTLY OF THEIR BASE CONTENT FOR DNA SEQUENCING BY HYBRIDIZATION.

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The possibility of equalizing DNA duplex stability is imperative for the application of sequencing by hybridization. We are developing a new strategy to obtain DNA duplexes having a thermal stability independent of their base content. Our approach consists in the modification of one of the four natural deoxynucleosides to form with complementary nucleoside a base pair having a stability very close to that of the other base pairs. Modified nucleic bases have been designed and incorporated into oligonucleotides. The influence of these modifications on duplex stabilities has been studied by absorption spectroscopy thus allowing the selection of N-4-ethyldeoxycytidine ($d^{4\text{-Et}}C$) which hybridizes specifically with natural dG to give a $G^{4\text{-Et}}C$ base pair whose stability is very close to that of the natural AT base pair. Duplexes built with AT and/or $G^{4Et}C$ base pairs exhibit thermal stability independent of their base content in classical buffer solution which enables the control of the stability of DNA hybrids as a function of their length only.

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2-36

IMINO PROTON EXCHANGE AND BASE PAIR KINETICS IN THE AMP-RNA APTAMER COMPLEX. S. Nonin, F. Jiang & D. J. Patel

We report on the dynamics of base pair opening in the ATP-binding asymmetric internal loop and flanking base pairs of the AMP-RNA aptamer complex by monitoring the exchange characteristics of the imino protons. The exchange kinetics of the imino protons identify the existence of four additional hydrogen bonds stabilizing the conformation of the asymmetric ATP binding internal loop that were not detected by NOEs and coupling constants alone, but are readily accomodated in the previously reported solution structure of the AMP-RNA aptamer complex published from our laboratory. The hydrogen exchange kinetics of the non-Watson-Crick pairs in the asymmetric internal loop of the AMP-RNA aptamer complex have been characterized and yield apparent dissociation constants (aKd) that range from 10-2 to 10-7. Surprisingly, three of these aKd values are amongst the lowest measured for all base pairs in the AMP-RNA aptamer complex. Comparative studies of hydrogen exchange of the imino protons in the free RNA aptamer and the AMP-RNA aptamer complex establish that complexation stabilizes not only the bases within the ATP-binding asymmetric internal loop, but also the flanking stem base pairs (two pairs on either side) of the binding site. We also outline some preliminary results related to the exchange properties of a sugar 2'-hydroxyl proton of a guanosine residue involved in a hydrogen bond that has been shown to contribute to the immobilization of the bound AMP by the RNA aptamer.

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2-37

NMR SOLUTION STRUCTURE OF A *CIS*- AND A *TRANS*-PLATINATED CROSS-LINKED INTERSTRAND DNA. F. Paquet, C. Pérez, M. Boudvillain, J.-M. Malinge, M. Leng and G. Lancelot.

A 10 base pairs double-stranded oligonucleotide with the sequence d(CCTCG*CTCTC).d(GAGAG*CGAGG) containing a single interstrand cross-link resulting from chelation of the N7 position of two guanine residues on the opposite strands of DNA by a *cis*-diammineplatinum(II) residue was analyzed by ^1H NMR spectroscopy. Using all the NOESY and TOCSY data (208 constraints), we have obtained a solution structure of the cross-linked duplex by using the NMR-constrained molecular mechanics program JUMNA. The reversal position of the two cross-linked guanines placed the *cis*-diammineplatinum(II) residue in the minor groove. The stacking of the platinated guanines, the extrusion of the cytosines and the locally left-helix formation induced a bend of 40° toward the minor groove and an unwinding of 76°.

Similarly a 12 base pairs double-stranded oligonucleotide with the sequence d(CTCTCG*AGTCTC).d(GAGACTC*GAGAG) containing a single interstrand cross-link resulting from chelation of the N7 guanine and N3 cytosine by a *trans*-diammineplatinum(II) residue was analyzed by ^1H NMR spectroscopy and JUMNA (330 consraints). The cross-link induced a local alteration centered on G*C*: a syn conformation of the cross-linked guanine, an increased rise between these 3 base pairs and a weak buckle. The resulting helix was unwound and bent toward the major groove from 14°.

These structures are in accordance with results obtained by means of gel electrophoresis, chemical probes and footprinting experiments. This study indicates significant conformational differences in the interstrand cross-links produced in DNA by *cis*- and *trans*-$[Pt(NH_3)_2Cl_2]$ certainly in relation with the mechanism of antitumor effects of platinum drugs. Cisplatin is widely used for cancer chemotherapy while transplatin is clinically ineffective.

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2-38

BINDING OF THE HISTONE-LIKE MC1 PROTEIN TO BRANCHED DNA STRUCTURES **C. Paradinas, F. Toulmé, A. Gervais, J. C. Maurizot, F. Culard.**

MC1 is a small, basic, and relatively abundant protein present in various species of Methanosarcinaceae. By these characteristics, MC1 belongs to the class of proteins referred as histone-like proteins that compact DNA *in vitro* and that can play essential roles in different cellular processes. With respect to the characteristics of its primary structure and to its low α helix content, MC1 differs from eukaryotic histones and from the eubacterial histone-like proteins. MC1 preferentially binds to double-stranded DNA, as a monomer, in a non cooperative way and can protect DNA against thermal denaturation. The visualization of the complexes by electron microscopy has shown that MC1 induces sharp kinks into linear and circular DNA. Furthermore MC1 binding can introduce negative supercoils that can not be relaxed by a topoisomerase.

We have investigated the binding of MC1 to various branched DNA structures by gel retardation assays. MC1 selectively recognizes some of these structures. In that way, MC1 poorly binds to bulged DNA. Studies with molecules containing, 2, 4 and 6 bulged adenines show that the binding of MC1 to these DNA decreases with the number of extra adenines. On the other hand, MC1 preferentially binds to three and four-way junctions as compared with linear fragments of the same length. The binding is particularly high with the four-ways junctions and hydroxyl radical footprinting shows that MC1 binds to the sharp kinks of these junctions.

Clearly, the protein MC1 belongs to the family of proteins that both recognize and induce bends in the DNA.

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2-39

CHOLESTERIC MESOPHASE OF H1-DNA AGGREGATES.
J. Pelta, D. Durand, J. Doucet and F. Livolant

In Eukaryotic chromatin, H1 proteins are involved in the condensation of the nucleosomic fiber but also in the regulation of multiple functionnal properties of the DNA molecule. Although the aggregation of DNA by H1 histones has been largely investigated, the supramolecular organization of the condensates remained unsolved. Using small DNA fragments (146bp), we determined the conditions of precipitation of the DNA fragments as a function of H1 and NaCl concentrations. The structure of the aggregates, collected by centrifugation was investigated by polarizing and electron microscopy and also by X-ray diffraction analyses. The aggregates are highly fluid and two phases were found depending on the biochemical conditions: a cholesteric liquid crystalline phase and an isotropic phase. In the cholesteric phase, the intermolecular distances ranges from 41.3 to 44Å which corresponds to a concentration of about 205 mg/ml.

Comparison of this cholesteric H1-DNA phase with the pure DNA cholesteric phase reveals many differences, namely (i) a much smaller helical pitch (0.4 to 1 µm compared to 2.5 µm), which suggests that the protein induces a strong twist between DNA helices, (ii) a higher fluidity (iii) the presence of defects which were prohibited in pure DNA and (iv) an elongated shape of the germs, versus spherical concentric spherulites. We show that these differences make this DNA-protein phase a good model to describe the organization of procaryotic chromatin.

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2-40

A DIRECT MEASUREMENT OF THE LIFETIME OF DNA-ASSOCIATED WATER MOLECULES BY 1D NUCLEAR OVERHAUSER EFFECT.
A. T. Phan, J-L. Leroy and M. Guéron.

NMR provides crucial information on the dynamic aspects of water-nucleic acid interactions. NOE cross-peaks between DNA and water protons are positive, zero, or negative when the hydration water lifetime is respectively longer, equal to, or shorter than 0.5 ns ($\omega\tau$=1.12; proton frequency 360 MHz).

Methods: Using one-dimensional proton NMR we have measured NOE and ROE cross-peaks between water and DNA protons. The temperature was varied from -11°C to 50°C in order to determine the temperature at which the NOE cross-peak is zero, corresponding to a water residence time of 0.5 ns. The 1D mode improves the quality of NOE cross-peak normalization and the comparison of different experiments. Cross-peaks with DNA protons at the water frequency (H3') were eliminated with a spin-echo filter, taking advantage of their shorter T_2. Exchange-relayed peaks were cancelled out by selective irradiation of exchanging protons during the mixing time. Three deoxyduplexes are studied: $d(CGCGAATTCGCG)_2$ which is a benchmark for hydration studies, the B-DNA duplex $d(CGCGATCGCG)_2$ and the B'-DNA duplex $d(AAAAATTTTT)_2$.

Results: In all cases, negative cross-peaks from water to thymidine methyl and to many H8 protons indicate the presence of short-lived water molecules in the major groove. The cross-peaks from water to all A H2 protons are positive at low temperatures and negative at high temperatures. The temperature corresponding to null NOE is -5°C for $d(CGCGATCGCG)_2$, 15°C for the central A of $d(CGCGAATTCGCG)_2$ and 32°C for the two innermost A of $d(AAAAATTTTT)_2$.

It is noteworthy that the lifetime of the water molecules in the hydration spine of the $d(CGCGAATTCGCG)_2$ oligomer is only 0.5 ns at 15 °C.

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2-41

Morphology and structure of lipopolyamine/plasmide DNA complexes for gene therapy.
B. Pitard*, O. Aguerre-Charriol+, M. Airiau+ and J. Crouzet*.

Gene therapy is based on the vectorization of genes to target cells, and their subsequent expression. Cationic amphiphile-mediated delivery of plasmid DNA is the non-viral gene transfer method most often studied. We examined the supramolecular structure of lipopolyamine/plasmid DNA complexes under various condensing conditions. Plasmid DNA complexation with lipopolyamine micelles whose mean diameter was 5 nm revealed three domains as a function of the lipopolyamine/plasmid DNA ratio. These domains respectively corresponded to negatively, neutrally, and postively charged complexes. Transmission electron microscopy and X-ray scattering experiments on complexes originating from these three domains showed that their morphology depends on the lipopolyamine/DNA ratio, but that their particle structure consists of ordered domains characterized by even spacing of 80 Å, irrespective the lipopolyamine/DNA ratio. The most active lipopolyamine/DNA complexes for gene transfer were positively charged. They were characterized by fully condensed DNA inside spherical particles (diameter: 50 nm) sandwiched between lipid bilayers.

The present results show that supercoiled plamsid DNA is able to transform lipopolyamine micelles into a supramolecular organization characterized by ordered lamellar domains.

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2-42

SPECTROSCOPY AND MOLECULAR PROPERTIES OF THIAZOLE ORANGE-OLIGONUCLEOTIDES COMPLEXES.
S. Prodhomme, J.Ph. Demaret, M. Egret-Charlier, L. Morin-Allory, U. Asseline, M. Chassignol, S. Vinogradov, N.T. Thuong and P. Vigny.

Purpose : Design and characterisation of new labeled oligonucleotides with enhanced fluorescence when complexed to their complementary targets.

Methods : Fluorescence spectroscopy and molecular modeling have been used to characterise non covalent thiazole orange (TO) - nucleic acids complexes. TO has also been attached to various 14-mers oligodeoxynucleotides *via* polymethylene chains linked at the 5' position and studied by the same techniques.

Results : TO exhibits a very low fluorescence quantum yield when free in aqueous solution (ϕ=2.10^{-4}). Its fluorescence quantum yield remarkably increases when intercalated in ds-DNA (ϕ=0.3), whereas it is only 10^{-3} when interacting with ss-DNA. Chemical linkage of TO to ss-DNA induces a fluorescence increase of one or two orders of magnitude due to the folding of the strand around TO. Despite the rather large size of TO, the base pairing with the complementary sequence is still possible, although no evidence for intercalation has been found as deduced from quantum yields and models. Furthermore, models have shown the influence of the length and the linking position of the polymethylene chain on the intercalation mechanism : a minimal length of 14, 12 or 10 carbons is required for linking at the 5'-terminus, 3'-terminus or internucleotidic positions, respectively.

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2-43

ENANTIOMERIC DEOXY-L-NUCLEOTIDES STABILIZE Z-DNA. S. vichier-Guerre and B. Rayner.

The conformational heterogeneity of DNA has been established. Left-handed Z-DNA represents a major conformational change which can exits *in vivo* although its precise role remains to be identified. Different conditions are known which stabilize Z-DNA *in vitro*. They include alternating purine-pyrimidine sequences, inclusion of 5-methylcytosine (^{Me}C) or 9-methylguanine, use of high salt concentration. In addition, a non-Z left-handed conformation, mirror-image of right-handed B-DNA, was evidenced in enantio-DNA, which was synthesized chemically from enantiomeric 2'-deoxy-L-nucleotides. We addressed the question whether the introduction into a short DNA duplex amenable to Z conformation of few unnatural 2'-deoxy-L-nucleotides, prone to induce a left-handed conformation, might contribute to stabilize Z conformation. For that purpose, a self-complementary decadeoxynucleotide d(^{Me}C G ^{Me}C G ^{Me}C G ^{Me}C G ^{Me}C G) was synthesized in which the central ^{Me}C and G were substituted by enantiomeric L-C and L-G respectively. Circular dichroism signals at 255 and 295nm were used as markers of B and Z conformations respectively. The favorable effect on Z conformation resulting from the introduction of two L-nucleotides was evidenced by two different ways: a) a substantially lower NaCl concentration (0.65 instead of 1.05 M) is necessary to induce 50% transition from B to Z conformation and b) a higher thermal stability of Z duplex at 3 M NaCl. In conclusion, despite the unability of enantio-DNA to adopt a left-handed Z conformation, constitutive enantiomeric deoxy-L-nucleotides, when incorporated in DNA, can contribute to stabilize Z-conformation.

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2-44

MEASURING DNA-STACKING FREE-ENERGIES WITH A CHEMICAL NUCLEASE: A THERMODYNAMIC ANALYSIS OF DNA-CLEAVAGE PATTERNS BY BIS(1,10-PHENANTHROLINE)-COPPER ION. F. Schaeffer.

Bis(1,10-phenanthroline)-copper(I) ion (OP_2Cu^+) binds reversibly to B-DNA and makes single-stranded cuts by oxidative attack on the deoxyribose moiety. The deoxyribonuclease activity is sequence dependent yet not nucleotide specific at the cutting site. OP_2Cu^+ sequence specificity was analysed in terms of local variations of DNA stability. Kinetic constants of strand cleavage were measured at sequence positions on the two strands using a gel electrophoresis technique and converted into activation free energies of the cleavage reaction. DNA unwinding free energies were calculated from the base sequence using B-DNA stacking parameters determined from high resolution UV-melting experiments. The two free energy variations were statistically compared along the DNA sequences of a series of DNA restriction fragments bearing the binding sites of regulatory proteins. This study shows that in B-DNA, the mean activation free energy of strand cleavage at couple of opposing sugars across the minor groove varies statistically quantitatively like the unwinding free energy of the DNA sequence delimited by the opposing sugars (3-4 bp). Using the same approach, it is shown here that DNA cleavage kinetics by OP_2Cu^+ can be used to measure DNA base-pair stacking free energies at T-tracts which adopt the B' format. Reference: Schaeffer F, Rimsky S, Spassky A (1996) J Mol Biol 260: 523-539.

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STRUCTURE-DEPENDENT RADIOLYSIS, RADIOSENSITIZATION AND RADIOPROTECTION OF DNA. M. Spotheim-Maurizot, L. Tartier, C. Savoye, M. Begusova, S. Hugot, D. Sy, R. Sabattier, V. Michalik and M. Charlier.

Using sequencing gel electrophoresis, we have shown that the radiation-induced DNA strand breakage probability at a given nucleotide site depends on structural variations due to sequence, topological stress (supercoiling), and binding of ligands (proteins, polyamines, metal ions, drugs). The initial event that leads to a strand break is the abstraction of the H4' and H5' atoms of deoxyribose by an OH· radical issued from water radiolysis. These atoms are located in the minor groove of B-DNA.

For instance, in a naked DNA, some sequences (e.g. the bent 5'-AATT regions) are less radiosensitive than "random" DNA, because of the reduced accessibility of H4' and H5'2 in the narrow minor groove of these sequences. In an alternate $(GC)_n$ sequence, all G's are more radiosensitive, and all C's are more radioresistant in a supercoiling-induced left handed Z-DNA than in the right handed canonical B-DNA. This can be explained by the low accessibility of the H4' of the C and the high accessibility of this atom of the G in the Z-DNA. In B-DNA this accessibility is the same at G and at C. The transition metal ion Cu^{2+} increases (by radiation-induced Fenton type reactions) the breakage at runs of Pu, at Py located 5' from a Pu or at runs of Py flanked by Pu, sequences with natural or ion-induced particular structures. The thiol and the disulphide derived from the radioprotective drug WR-2721 (marketed as Ethyol-Amifostine), reduce DNA strand breakage of some sequences and do not protect (or even sensitise) other sequences. On the basis of molecular modelling results we explain these sequence-dependent radiomodifying effects by unlike structural variations induced by the electrostatic binding of the drugs to different sequences.

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2-46

CONFORMATIONAL STUDY OF THE HIV ACTIVATION DOMAIN, THE DNA SITE BINDING THE TRANSCRIPTION FACTOR NF-κB C. Tisné, B. Hartmann$, E. Hantz, C. Simenel, M. Delepierre

The DNA sequence within the HIV-1 Long Terminal Repeat (LTR) that responds to T-cell activation signals, is a region containing two direct repeats of a ten base pair motif called κB. Initiation of transcription occurs only if these DNA sequence motives are occupied with the transcription factor NF-κB. The mutation of the three base pairs GGG in CTC of the κB site prevents from binding of NF-κB.

The solution structure of two DNA non-palindromic duplexes of 16 base pairs related to the 3' NF-κB binding site were analysed by proton and phosphorus two-dimensional NMR and molecular modelisation. The first sequence corresponds to the wild type and the second sequence contains the specific mutation that abolishes the NF-κB binding. Assignments of all proton and phosphorus resonances show that only the structure of the nucleotides around the mutation is affected by it. In addition, a detailed analysis of NMR data exhibits significant local structural differences between the two duplexes that bring elements to explain their different behaviour towards NF-κB binding. The modelisation achieved using the NMR data emphasizes these differences.

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2-47

DNA Curvature Detected by Fluorescence Techniques

G.Chirico, M.Collini, J.Langowski, V.Sauermann, K.Tóth

Most indications for sequence-induced DNA curvature come from gel migration anomaly (GMA), electron microscopy (EM) and through cyclization kinetics of DNA fragments. Both in GMA and EM, external factors may influence the structure of the fragments to be examined. Using fluorescence techniques, we could demonstrate the curvature in free solution of DNAs containing A-blocks repeated in phase with the helix pitch.

The first technique, fluorescence resonance energy transfer (FRET), measures the average distance of two dyes attached through linkers to the two ends of the DNA. The maximal distance detectable by FRET being 10nm, we constructed fragments of only 3 helix turns (31bp) containing A_6 blocks. Measurements proved qualitatively the ≈7% change in FRET predicted from curvature models for this sequence, compared to straight sequence of the same base composition. Optical melting and circuar dichroism were used to confirm that the fluorescence label caused no major structural change in the DNA.

For longer fragments and smaller relative curvature, we applied fluorescence polarisation anisotropy (FPA) in a phase fluorimeter. This method measures the decay of fluorescence polarization from ethidium intercalated randomly between the base pairs (≈ 1/100bp). The polarisation decay depends on the rotational diffusion constant of the DNA fragment; curved DNA is expected to exhibit a slower rotation about the long axis. FPA measurements on 100 bp fragments containing 8 repeats of A_1, A_3, A_4 and A_5 show that in solution permanent curvature occurs for repeats of A_4 or larger.

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3-1

THE ROLE OF NOVEL ACTA-LIKE PROTEINS IN ACTIN ORGANISATION AND MOVEMENT OF HUMAN CELLS.
R.M. Golsteyn, M.C. Beckerle, D. Louvard and E. Friederich.
The organisation of actin by the pathogenic intracellular bacteria *Listeria monocytogenes* is similar to that at the plasma membrane of eucaryotic cells. *L. monocytogenes* express ActA protein at their surface which enables them to move in host cells by recruiting eucaryotic actin. We used anti-ActA antibodies to identify ActA-like proteins in human cells by the methods of immunofluorescent localisation, 2D-gel electrophoresis, and production of candidate proteins by transient transfection. We found that human zyxin, a component of the actin cytoskeleton, has structural features similar to ActA. Zyxin and ActA exhibit a common 300 amino acid proline/glutamate rich domain that, in *L. monocytogenes*, is important in actin dependent movement. To compare the function of zyxin with ActA we tagged the proteins with a inner plasma membrane localisation sequence (a CAAX box) and produced these constructs by transient transfection. We found that the proline rich domain of zyxin disrupted the actin cytoskeleton and cell shape in a similar manner as the proline rich domain of ActA. We reproduced an ActA phenotype by transfecting cells with a chimera composed of the N-terminal domain of ActA with the proline/glutamate rich domain of zyxin. Zyxin-CAAX production resulted in the displacement of VASP from focal adhesions. Furthermore, zyxin and ActA both bound to Ena/VASP protein family members *in vitro*. In view of the structural and functional similarities of the domain common to ActA and zyxin, we propose that zyxin acts as a module that enhances actin organising activity of different sites in cells by delivering actin monomers.

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3-2

REGULATION OF THE TRACTION FORCES EXERTED BY LOCOMOTING KERATOCYTES
K. Jacobson*, T. Oliver*, M. Dembo**, E. de Beus*, J. Lee*.

The graded radial extension [GRE] model is a kinematic description of the close coordination of extension and retraction of the cell margin required for fish epidermal keratocytes to exhibit a rapid, gliding mode of locomotion. In this study, we investigated the traction forces generated by the cell and how they depend on actomyosin interactions, $[Ca^{++}]_i$ and cell adhesion. To image traction forces, a silicone rubber film assay was developed that allows measurement of the distribution of traction stresses and their magnitudes. The dominant and unexpected features of the traction force pattern are the strong, equatorial, actomyosin dependent tractions that are oriented perpendicular to the direction of locomotion and the lack of propulsive [rearward directed] tractions near the extending leading margin. These traction
patterns change dramatically when keratocytes become tetherd or turn sharply. Ca transients are measured when the cell becomes stuck and intracellular Ca release induced by photoactivation stimulates retraction. One view of the strong equatorial tractions is that they are required for the massive coordinated retraction of the rear margin observed for rapidly moving keratocytes. Transient Ca increases initiated through stretch activated Ca channels are associated with increased contractility and/or 'deadhesion' required to complete retraction when the cell gets 'stuck'.

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3-3

PLASMA MEMBRANE ORGANIZATION BY THE MEMBRANE SKELETON AS STUDIED BY SINGLE PARTICLE TRACKING AND LASER TWEEZERS.
A. Kusumi.
Movements of transferrin and α_2-macroglobulin receptor molecules in the plasma membrane of cultured NRK cells and of band 3 in human erythrocyte ghosts were investigated by single particle tracking with a time resolution of 0.2 ms by labeling the proteins with colloidal gold particles. Their movements suggest that the plasma membrane is compartmentalized into many small domains of $\approx$ 0.25 μm^2 (0.01 μm^2 for band 3) with regard to lateral diffusion of many membrane proteins and that these molecules move from one compartment to an adjacent one every 25 s (200 ms for band 3) on average (hop diffusion). Transferrin receptor (TR)-particle complexes were dragged laterally along the plasma membrane using laser tweezeres. The majority (90 %) of TR which exhibited hop diffusion could be dragged past the intercompartmental boundaries by laser tweezers at a trapping force of 0.25 pN. At a dragging force of 0.05 pN, half of the TR molecules escaped from the laser trap at the boundaries, and such ecape occurred in both the forward and backward directions of dragging. The boundaries are elastic with an effective elastic constant of 1-10 pN/μm. These results are consistent with the proposal that the compartment boundaries consist of membrane skeleton (membrane skeleton fence model). Remaining TR ($\approx$ 10 %) exhibited binding to elastic structures.

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3-4

ADHESION AND DYNAMICS OF VESICLES.
U. Seifert.
Fluid bilayer vesicles are a well-defined model system for the study of more complex cells. Their configurations are determined by the interplay between curvature energy, geometrical constraints, direct and fluctuation-induced interactions and hydrodynamic dissipation (1). For adhesion of a vesicle to a substrate, a comprehensive description requires both a theory of its macroscopic aspects (such as shape, effective contact angle) and an understanding of the mesoscopic thermal fluctuations of its bound part. These quantities can be calculated whithin a recent theory that combines these two aspects self-consistently (2). The dynamics of vesicles will be illustrated with the results of a theoretical study for the shape transformation induced by shear flow (3). he stationary state of the vesicle in shear flow depends on its area to volume ratio but not on the shear rate. Thus, shear is a singular perturbation. For the tank-treading motion of the fluid membrane in the stationary state, we obtain the frequency as a function of shear rate and reduced volume of the vesicle.

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3-5

CELL ADHESION STUDIED BY FLUORESCENCE INTERFERENCE ON SILICON. D. Braun, P. Fromherz.

Fluorescence interference-contrast microscopy [1] is used to measure the distance of cell membranes from silicon dioxide on silicon chips. The cell membrane is stained with a fluorescent dye. The label interacts with the standing modes of light in front of the silicon surface. As a result the fluorescence intensity depends on the position of the membrane. An internal scaling of fluorescence intensity is obtained by attaching a cell to microscopic steps of silicon dioxide. The variation of fluorescence intensity as a function of the height of these steps is fitted by an optical theory which takes into account the interference of exciting light and of emitted light in a microscope. As a test system we used the ghosts of erythrocytes attached to silicon dioxide with poly-lysine [2]. The distance from the outer surface of the membrane to the substrate is about 5.5 nm, corresponding to the thickness of the glykocalix. The adhesion region is smooth. We studied glia cells and neurons from the hippocampus of the rat cultivated on laminin. The distance of the membrane from the substrate is about 100 nm.

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3-6

SPECTROSCOPIC CHARACTERISATION OF THE MOLECULAR PROCESSES OF CELL ADHESION

C-W Chung, A M Hutchinson and R M Cooke

Many inflammatory processes result from the abnormal recruitment of cells such as neutrophils or eosinophils. The processes of recruitment usually involve cell adhesion and activation, which thus represent attractive targets for therapeutic intervention. Several classes of molecules participate in these events and this talk will focus on three of them: selectins, chemokines and integrins. Each interacts with a peptide or carbohydrate epitope as part of the molecular recognition process. NMR studies of E-selectin have enabled the bound conformations of a carbohydrate and peptide ligands to be analysed. The solution structure of the chemokine RANTES has also been determined by NMR, enabling modelling of the interaction with its receptors. The binding of I-domains, key motifs within neutrophil integrins, to their biological partners has been monitored by surface plasmon resonance, allowing the roles of protein, carbohydrate and cations in the adhesion process to be defined.

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3-7

DO ENDOCYTIC VESICLES MOVE ON ACTIN FILAMENTS? M.N. Cordonnier, D. Louvard, E. Coudrier.

It has been recently shown that actin filaments participate in the organization and the function of endocytosis in a mouse hepatoma cell line (BWTG3) (Durrbach et *al.*, 1995, JCS, 109:457). The depolymerisation of actin filaments by cytochalasin D inhibits two steps of endocytosis: 1) uptake of ligands and 2) delivery of ligands to the degradative compartment. Furthermore, the production of the truncated chicken brush border myosins-I (BBMI) in these cells affects the delivery of ligands to the degradative compartment (Durrbach et *al.*, 1996, PNAS, 93:7053). Altogether, these data suggest that an acto-myosin driven mechanism is involved in this step of endocytosis. It is likely that an endogenous myosin-I related to BBMI might bind transiently the juxtanuclear endosomes on actin filaments and thereby regulate the delivery of ligands to the degradative compartments. We have observed that endosomes prepared from BWTG3 cells according to the protocol of Gorvel et *al.* (1991, Cell, 64:904) are enriched similarly for endocytic markers such as transferrin receptors and Rab 5 as well as a protein immunologically related to BBMI. We have also detected this protein in a lysosomal fraction prepared according to the protocol of Green et *al.* (1987, JCB, 105:1227). Furthermore, the endosomal fraction is able to bind actin filaments immobilized in vitro in a flow-cell in an ATP-dependent manner. We are presently studying the behaviour of the lysosomal fraction in the same test and whether non functional BBMI protein that affect endocytosis can compete for this interaction. We also attempt to set up an in vitro assay to study the movement of endosomes on actin filaments.

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3-8

MEASURING THE IN VIVO BENDING MODULUS AND ADHESION ENERGY OF MOTILE CELLS USING SMALL SHEAR FORCES. R. Simson and E.Sackmann.

Combining a parallel plate flow chamber and Reflection Interference Contrast Microscopy (RICM), we determined the elastic properties of various strains of *Dictyostelium discoideum* . RICM together with digital image processing allows reconstruction of the cell contour to a height of about 1 μm. We calculated tension and bending energy as well as the adhesion energy of a cell by analyzing the changes in the cells contour due to a laminar flow. The applied shear stress (0.4 to 1.2 Pa) was large enough to probe the elasticity of the cell, yet small enough to prevent detachment. In control experiments on red blood cells we found a bending energy of $\approx 10\ k_BT$ in good agreement with previous results. For wild type *Dictyostelium discoideum* we found a bending energy of $\approx 400\ k_BT$. Markedly reduced values of about 100 k_BT were found for mutants lacking important actin binding proteins. Mutants with a disturbed coupling between cytoskeleton and lipid bilayer also exhibited a reduced adhesion energy.

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3-9

MIMICKING CELL ADHESION BY THE INTERACTION OF SUPPORTED MEMBRANES AND GIANT VESICLES FUNCTIONALIZED WITH THE HOMOPHILIC RECEPTOR PROTEIN CONTACT SITE A (csA)

(A. Behrisch, A. Kloboucek, G. Gerisch*, E. Sackmann)

The contact site A glycoprotein is a developmentally regulated homophilic cell adhesion molecule from *Dictyostelium discoideum*. A model system for cell adhesion based on specific receptor interaction is established. The biological membrane is mimicked with phospholipids and the glycocalyx with lipopolymers. Applying various techniques (film balance, micro fluorescence, FRAP), the purified GPI-anchored protein was reconstituted in solid supported bilayers and giant vesicles consisting of uncharged phospholipids and PEG-lipopolymers. Interaction of the functionalized giant vesicles with the supported bilayer was observed with a Reflexion Interference Contrast Microscope (RICM). This technique allows quantitative studies of surface profiles near the contact area. Local adhesion energies can be calculated from the interference pattern by analyzing the height profile S(x) near the contact line in terms of the tension equilibrium (Young equation). Depending on the protein concentration, lateral phase separation leading to the formation of domains of tight adhesion (adhesion plaques) separated by areas of weak adhesion exhibiting pronounced flickering can be observed. Adhesion energy is shown to be larger up to three orders of magnitude for adhesion plaques than for the weakly adhering regions.

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3-10

MECHANOOSMOTIC COUPLING IN THE MOTILE HELICAL BACTERIUM *SPIROPLASMA MELLIFERUM*.

L. Béven and H. Wróblewski.

In spite of being devoid of cell wall and flagella, spiroplasmas possess a well defined helical cell shape and are motile. These tiny prokaryotes swim in liquid media by a screw-like mechanism and change direction by bending, flexing, and twitching movements in response to chemical stimuli. In contrast to bacteria propelled by rotating helical flagella, almost nothing is known about the mechanisms governing cell shape and motility in spiroplasmas. During the exponential growth phase, *Spiroplasma melliferum* cells are helical : length, 6.5 ±1.6 µm ; helix diameter, 0.9-1.0 µm ; pitch, 0.8 ±0.1 turn/µm. When energized by glucose at e.g. neutral pH, the cells build up a proton-motive force (Δp) of -77 ±7 mV (inside negative) with a major contribution of the electrical transmembrane potential ($\Delta\Psi$ = - 68 ±5 mV). Using different ionophores, we show that cell helicity is maintained if -108 ±9 ≤ Δp ≤ -69 ±6 mV (-99 ±8 ≤ $\Delta\Psi$ ≤ -59 ±5 mV). Hyperpolarisation of the membrane induces a relaxation of the cell helix whereas depolarisation triggers a cascade of deformations : (1) loss of helix flexibility, (2) helix stretching , (3) helix relaxation and formation of aneurisms, (4) swelling of the aneurisms, and (5) splitting of the cells into several rounded vesicles. It should be noted that motility has the same dependence on the proton-motive force than cell shape, but motility is always lost first upon alteration of Δp or $\Delta\Psi$. The observed phenomena being independent of the consumption of ATP, we conclude that in spiroplasmas, helical cell shape and motility are tightly coupled to the transmembrane electrochemical potential.

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3-11

DYNAMIC OF THE ACTIN-MYOSIN INTERFACE STUDIED BY CHEMICAL CROSS-LINKING.

J. Van Dijk, C. Fernandez & P. Chaussepied.

The dynamic of the actin-myosin interaction plays an active role during muscle contraction and the actin-linked cell motility processes. This dynamic is revealed by a multistep binding process directly linked to the hydrolysis of ATP by myosin, i.e. to the nature of the nucleotide in its active site (Geeves & Conibear 1995, Biophys.J. *68*, 194). In order to identify the changes in the actin-myosin interface during these steps, we have undertaken chemical cross-linking experiments in the presence of various nucleotide analogues. The gel electrophoresis pattern of the cross-linking reaction induced by EDC was found to be sensitive to the nucleotide bound to the actin-myosin head complex. In the absence of nucleotide or in the presence of ADP, four cross-linked products with apparent MWs of 165, 175, 200 and 265kDa were obtained while with the ADP.Pi analogues (ADP.BeF_x, ADP.AlF_4 and ADP.VO_4) a 265kDa band was produced almost exclusively. When ATP was added, the major cross-linked product was the 200kDa band. The time course analysis of these cross-linking reactions together with the estimation of the amount of complex showed that these differences were more likely due to changes in the actin-myosin interface. It was previously found that the 165 and the 175kDa bands are composed of one myosin head cross-linked to the stretch of residues 1-4 of one actin monomer while the 265kDa band contains one S1 bound to the same residues of two adjacent actin monomers (Bonafe & Chaussepied, 1995, Biophys.J. *68*, 35). Taken together, these results led us to propose a new model for the mechanochemical cycle in which the the myosin head interacts with the N-terminus of two actin monomers to stabilise and correctly orient the pre-working stroke actin-myosin-ADP.Pi complex.

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3-12

CHEMICAL AND ELECTRICAL GUIDANCE OF HIPPOCAMPAL NEURONS.

S. Dertinger, P. Fromherz.

The outgrowth of dendrites and axons has to be controlled in order to study signal processing in individual vertebrate neurons [1] and in simple neuronal nets. In previous studies we used chemical patterns to obtain defined arborization of neurites from leech neurons [2]. It is the goal of the present study to combine chemical patterns with local electrical stimuli to prepare defined dendritic trees of rat neurons in culture. On one hand we develop reusable hydrophobic patterns with selectively adsorbed laminin. Extended growth of dendrites in dilute culture was obtained. On the other hand local voltages are applied by microelectrodes which affect the shape of the growth cone and direct its growth. The electrical guidance was found to depend on the conditions of the medium and the polarity of the voltage. The study on the combined effect of electrical and chemical guidance is in progress.

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3-13

THE RENEWAL OF THE EPIDERMIS: A TOPOLOGICAL MECHANISM B. DUBERTRET, N.RIVIER

Using a topological approach, we study the dynamics of the basal membrane of the mammalian epidermis when basal cells detach or divide. A theoretical characterization of the steady state of the tissue, in very good agreement with experimental data, includes for the first time the division and the disappearance of cells in a two-dimensional random cellular structure. We predict a strong correlation between the size of the attachment of basal cells to the basal membrane and their biological behavior (division or detachment). This suggests that the main factor determining the fate of basal cells, and thus controlling the renewal of the epidermis, is the cells' surface tension and adhesion.

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3-14

KINETICS OF MOLECULAR RECOGNITION: DISSECTING THE PATHWAY OF COLCHICINE BINDING TO TUBULIN.

C. Dumortier[1], J.L. Potenziano[2], S. Bane[2] and Y. Engelborghs[1]

The pathway of drug binding to proteins can be dissected by studying the kinetics of the binding of analogs, and by competition kinetics with constituent smaller molecules. 2-Methoxy-5-(2',4'-dimethoxyphenyl)-2,4,6 cycloheptatrien-1-one (A'C) is a A-ring analogue of MTC, in which one methoxy is replaced by a hydrogen atom. MTC [2-methoxy-5-(2',3',4'-trimethoxy)2,4,6-cycloheptatrien-1-one] is a colchicine analogue which lacks the B-ring. This work describes the spectroscopic and kinetic features of A'C binding to tubulin. Binding is accompanied by a strong enhancement of A'C fluorescence and quenching of protein fluorescence. The kinetic and thermodynamic parameters were obtained from fluorescence stopped flow measurements. The observed pseudo-first-order rate constant of binding increases in a nonlinear way, indicating that this ligand binds in two steps: a fast initial binding of low affinity followed by a slower isomerisation step leading to full affinity. The pathway of A'C is shown to differ considerably from that of MTC binding. Since its structural difference is located in ring A, this result points to its use of ring A in the first step.

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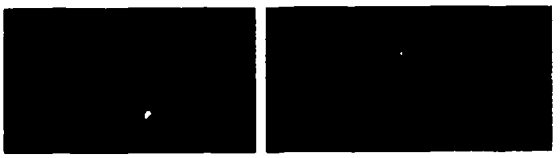
3-15

FLUORESCENT TAXOIDS AS PROBES FOR MICROTUBULE AND CENTROSOME TARGETS. J.A. Evangelio, M. Abal, A.A. Souto, I. Barasoain, M.P. Lillo, A.U. Acuña, F. Amat-Guerri and J.M. Andreu.

7-(N-fluorescein-L-alanyl)taxol (FLUTAX) is a member of a series of fluorescent derivatives of the microtubule-stabilizing antitumour drug taxol (Souto et al., Angew. Chem. Int. Ed. Engl. 34, 2710-2712, 1995). FLUTAX induces the assembly of purified GDP-liganded tubulin into microtubules. One molecule of FLUTAX binds per each assembled αβ-tubulin dimer, competitively with taxol, with eight-fold weaker apparent affinity. The absorption spectrum of FLUTAX is modified upon binding to microtubules and the apparent pK of fluorescein (6.54) shifts down approximately 0.4 pH units. The time-resolved fluorescence anisotropy changes observed are compatible with the immobilization of the taxoid moiety in the microtubule binding site. FLUTAX-induced microtubules can form bundles and aster-like structures which are readily observed with the epi-fluorescence microscope. When given to diverse permeabilized cells, FLUTAX specifically binds to microtubules and centrosomes, which constitutes a new procedure to directly visualize these subcellular structures (see Figure). 80 nM FLUTAX inhibits the cell cycle of U937 human monocytic leukaemia cells and triggers apoptosis. Fluorescence microscopy observations of these cells show FLUTAX binding to centrosomes, and more weakly to centrosomal and cortical microtubules, giving by first time direct clear images of specific binding of a taxoid to its subcellular targets in living cells.



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3-16

SELF-GATING OF ION CHANNELS IN CELL ADHESION ?

P. Fromherz.

When a cell membrane is in close contact to an inert surface, any current through the membrane is forced to flow along the thin cleft between membrane and surface. The concomitant voltage-drop in the cleft changes also the voltage across the membrane. Voltage-dependent processes in the membrane may be affected such as electrophoresis [1] and gating of ion channels. This coupling of adhesion and gating is investigated here in two steps [2]: (i) Current and voltage are described by a simple electrical circuit. The model is solved analytically. The usual smooth gating is transformed to switching with bistability, hysteresis and memory. The transition between states of low and high membrane conductance is triggered by small changes of the strength of adhesion or of the membrane potential. (ii) The adhesion region is described as a two-dimensional cable. The nonlinear Kelvin-type equation is solved numerically. This computation confirms the simple model in all aspects: The common biophysics of membranes implies a novel type of instability in the geometry of adhesion.

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3-17

A POSSIBLE PHYSICAL MECHANISM OF RED BLOOD CELL VESICULATION AT HIGH pH. A. Iglic[1,2], H. Hagerstrand[3], M. Bobrowska-Hagerstrand[3] and V. Kralj-Iglic[2]

The membrane of human red blood cells is essentially composed of two parts, the lipid bilayer and the protein skeleton. The skeleton is attached to the inner side of the bilayer mainly by bilayer integral proteins. The normal resting shape of red blood cells at physiological pH 7.4 is the discocyte. However, at alkaline pH $\simeq$ 11 the shape of red blood cells is composed of a spherical parent cell and large spherical daughter vesicles. The daughter vesicles may be free or connected to the parent cell by a narrow neck. We show in this paper ghat red blood cell shapes at pH $\simeq$ 11 correspond to some of the calculated limiting shapes of a closed lipid bilayer having an extreme area difference between the outer and the inner monolayer. Therefore, it is suggested that the observed shapes of red blood cells at ph $\simeq$ 11 are caused by abolishment of the skeleton-bilayer interactions at this pH.

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3-18

VOLTAGE-PROFILE IN ADHESION SITE MEASURED BY TRANSISTORS. V. Kiessling, B. Müller, P. Fromherz.

In cell adhesion a thin sheet of electrolyte is insulated laterally from the cell plasma by the cell membrane and kept on bath potential at its periphery. The system forms a core-coat conductor or 2D-cable [1]. It is expected that ohmic and capacitive currents through the membrane give rise to a voltage drop in the extracellular space. In a first attempt a row of eight transistors was placed beneath a large nerve cell to detect this voltage-profile [2]. To prove the validity of the cable concept quantitatively we modified the set-up in two aspects: (i) We developed a chip with 96 transistors closely spaced in a row and (ii) we chose a simpler membrane as giant erythrocytes made by electrofusion. AC-voltages from 1 to 5000 Hz are applied through a patch-pipette to the attached cells. The response of the transistors is detected in amplitude and phase. The data are fitted with solutions of the 2D-cable equation. As a result we obtain the electrical parameters of the membrane in the attachment region and the sheet resistance of the adhesion site.

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3-19

FLICKER SPECTROSCOPY OF ERYTHROCYTES: EXPERIMENT VERSUS NUMERICAL SIMULATION. V.L. Kononenko

Frequency spectra of the stochastic bending oscillations of erythrocyte membrane (flicker phenomenon) were measured in a broad range 0.05÷500 Hz using two optical regimes: phase contrast and backward laser light scattering, both for a single cell and for rouleaux. An approximate but comprehensive theory of the flicker was developed which takes into account the influence of the cell shape on the discrete set of the membrane bending modes, and the instrumental distortion of the intrinsic shape of the flicker spectrum. The computer model of the Flicker Spectroscopy (FS) was developed, which included the numerical simulation of three main stages of FS: thermal excitation of the intrinsic spectrum of cell membrane undulations, optical-electronic processes of the detector signal formation, and the mathematical procedure of the acquisition, Fourier analysis and statistical evaluation of the signal samples. It is accepted in the literature that various alterations of the shape of flicker spectra are due to the change of mechanical properties of erythrocyte. The numerical simulation showed very strong influence of three other types of parameters: geometrical (thickness/diameter ratio of a cell), instrumental (type of the optical regime, size of the measuring diaphragm projection at the cell h), and data-processing (type of the spectral window, background noise level, zero-frequency amplitude). It is shown, that the change in the cell shape, rather then in its mechanical properties, accounts for the observed variation of the effective spectrum width $\Delta\omega$ with the osmotic pressure. The accuracy of $\Delta\omega$ determination better then 10% requires h<1 μm. It is shown, that FS can be used to monitor erythrocytes mechanical properties only in combination with the cell geometry monitoring. However, the $\Delta\omega$ value can be measured by FS rather accurately. So, it can be used as a generalized index of the cell's geometromechanical status related to its physiological state.

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3-20

CORRELATION BETWEEN QUASI PERIODIC INTRACELLULAR CALCIUM AND MOTION PARAMETERS OF MIGRATING NEUTROPHILS N. Korb, L. Köhler, M. Eberhardt, H. Gruler

Amoeboid cells like granulocytes can be regarded as self-organized machines [1,2]. Our model yields a signal transduction chain that describes as:

1) A weak cellular input signal originating from membrane-bound receptors occupied by molecules that stimulate migration steers a large flux of energy and mass.

2) A chemical amplification stage that produces the strong second intracellular signal. Several functions are performed by this second intracellular signal like the activation of the microfilaments (linear motor) and the renewal of the membrane-bound receptors. The goal of this work is to find out wheather intracellular calcium as an important intracellular messenger is involved in this process. For this purpose we use calcium sensitive fluorescence microscopy in combination with phase contrast microscopy to get both, the calcium distrubution within the cell and its shape at the same time. (Utilization of granulocytes from heparinized venous blood of healthy human donors by standard methodes.)

We find that integrated fluorescence intensity shows quasi periodic fluctuations with a characteristic time of $\approx$ 1 min. This characteristic time shows only small variations for cells from one and the same donor (e.g. 60 ± 10 s) but is a function of the donors age and sex: It is shorter for women and old persons. Speed and shape of the cells correlate with the change in calcium concentration as predicted by our model. Our observations [3] of trajectories yield two independent times of 8 s for the time to amplify the input signal and $\approx$ 60 s as a persistence length for cells performing random walk. Our results show that calcium fluctuations are not responsible for the amplification step but might be important for the persistence of random walk.

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3-21

NEUTROPHIL LOCOMOTION IN A MICROPIPETTE.
V.Vereycken, C. Bucherer, J.C. Lelièvre and C. Lacombe.

Defined as a spontaneous displacement of a cell towards a chemical source, the cell motility is one of the most interesting phenomena of cell biology. In this work, mechanics with its principles and methods, is applied to cast a new light on unidirectional motion of the human neutrophil, which is the first cell operating in the immune response. A neutrophil is forced to migrate in a glass micropipette (inner diameter of 5 μm) in which is instituted by diffusion a concentration gradient of a chemoattractant molecule (fMLP). The experimental set-up is designed to observe the neutrophil displacement as a function of time. For 9.10^{-7} M initial concentration of fMLP and from microscopic observations, the mean speed and the mean force required to stop the neutrophil motion have been found to be respectively of 0.155 ± 0.04 μm/s for 68 neutrophils and 39 ± 4 nN for 9 neutrophils.

In a phenomenological point of view, the neutrophil behaves as a vehicule composed of a tractor and a trailor. The tractor has a linear traction-speed characteristic and the trailor containing the nucleus does not resist to the motion. Globular actin polymerisation inside the cell has been shown to be involved into the motion by using cytochalasin B. The mean speed of treated cells is tenfold less than the one of normal cells. This result has been confirmed by performing the neutrophil fragmentation.

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3-22

INTERACTION OF CARBOHYDRATE LIGAND INVOLVED IN CELL ADHESION.
T. Le Bouar, E. Joo, F. Pincet, E. Perez, F.Yvelin, J. Esnault, Y.M. Zhang, J.M. Mallet, P. Sinay.

Cell adhesion is of primary interest among many biological processes such as immune response or cell differentiation. The regulation of the interaction between two different cells through adhesion is driven by many receptor-ligand interactions which have each a specific role such as to slow down, to stop, or to attach to the other cell. Carbohydrates and especially sugar residues have been shown to play a recognition role in this process. It has been postulated that two Lewis-x groups have a specific interaction mediated by Ca^{++} ions. We study the homotypic recognition between trisaccharrides Lewis-x determinants with a surface force apparatus and functionnalized vesicles micromanipulations. These oligosaccharides constitute the headgroup of lipids that have been synthetized for the purpose of this study. We have demonstrated that a specific interaction occurs with calcium and will dicuss its mechanism.

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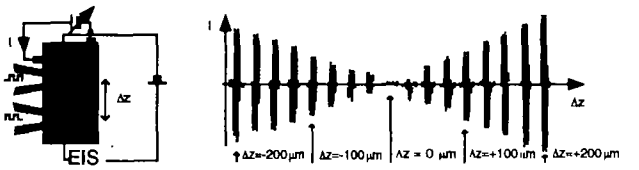
3-23

SPATIALLY RESOLVED SURFACE POTENTIAL MEASUREMENTS WITH THE LIGHT ADDRESSABLE POTENTIOMETRIC SENSOR (LAPS).
W.J. Parak, M. George, S. Kölblin, J.C. Behrends, G. ten Bruggencate, H.E. Gaub.

The LAPS is a semiconductor based device in electrolyte-insulator-semiconductor geometry (EIS) for measuring surface potentials. By



locally illuminating the silicon surface through the electrolyte with a pulsed light pointer, the local surface potential can be determined by measuring the amplitude of the photocurrent I. Figure 1 shows, how surface potential can be varied by a linear potential gradient between two additional electrodes. Using two 180° phase shifted light pointers, local surface potential differences can be measured. By varying the distance Δz between the light spots on the silicon surface, the shape of the potential gradient could be imaged. A future application is the measurement of membrane potentials of nerve cells adhered to the silicon surface. Figure 2 shows a neuron from rat brain grown on a laminin-coated LAPS wafer. In contrast to membrane potential recordings with field effect transistors, cells do not have to be fixed on special positions (gate electrode) but can grow freely on a completely unstructured surface, the point of measurement being selected with the light pointer.

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3-24

PATTERNS FORMATION IN CELL OR VESICLE ADHESION.

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Cell adhesion or vesicle adhesion is an important event for many cellular functions.The role of nonspecific forces on the adhesion of cell or vesicle on a support will be systematically studied. The presence of repulsive forces of different origins (electrical , hydration or steric) can stabilize the aqueous layer in the contact zone against rupture and lead to the formation of a new stable stationary state (periodic pattern). Nano-structures are observed in cell/cell or cell/solid support interactions (periodic contacts of the order of 1 μm) [1,2], or also in lipid vesicles adhering to a solid [3]. Recently, we analysed whether the emergence of such patterns is confirmed by a theoretical approach. A bifurcation analysis was performed [4] which allows to predict the existence of such structures and to determine the range of parameters for their occurrence. Numerical solutions of the nonlinear evolution equation allowed to observe new stable periodic patterns. Such patterns were already predicted in lipid bilayers [5]. Application to blister formation in electrostatically controlled adhesion of lipid vesicles will be discussed.

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3-25

^{31}P NMR SPECTRA OF ADP TIGHTLY-BOUND TO NCD AND KINESIN MOTOR DOMAINS. T. Shimizu[1,2], Y. Suzuki[3] and M. Tanokura[3].

The motor domains of kinesin and ncd contain ADP bound tightly at the enzymatic active sites even after purification. The micro-environments surrounding the phosphates of the tightly-bound ADP molecules in these motor domains were studied using ^{31}P NMR. The α- and β-phosphate of tightly-bound ADP molecule gave ^{31}P NMR signals at the positions shifted to higher and lower field, respectively, from those of free MgADP. This suggests that the electron density surrounding α- and β-phosphates of MgADP was likely to increase and decrease, respectively, upon formation of the complex. In addition, we found a difference between kinesin and ncd motor domains in the chemical shift of α-phosphate of bound MgADP, indicating that the electrostatic or magnetic microenvironments of this site of these motor domains differed from each other. When free MgADP was added at the same concentration to one of these motor domains, the peak areas of the α- and β-phosphate signals of bound MgADP were found to be much smaller than those of free MgADP. When the assay temperature was shifted from 20°C to 10 or 0°C, each signal of the bound MgADP exhibited a large temperature dependence in the peak area with both proteins. These results may suggest multiple conformations of these proteins depending on the temperature.

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3-26

THE CELLULAR LOCALIZATION OF THE INTEGRIN αvβ3 IS MODIFIED DURING ANGIOGENESIS IN VITRO. **B. Vailhé (1), P.Tracqui (2), X. Ronot (3,4), Y. Usson (3), F. Baffert (1), L. Tranqui (1).**

The aim of our work was to establish a correlation between the morphological differentiation of the human vein endothelial cells (HUVECs) on fibrin and the expression of the integrin αvβ3, which is the receptor involved in the cell adhesion on the fibrin matrix. Using time-lapse video microscopy, we studied the different steps which lead to the HUVECs reorganization into capillary-like structures (CLS). We observed that cells began to reorganize into "ring-like structures", and that the coalescence of such structures lead to the formation of CLS. The fibrin gel was distorted in places, suggesting that mechanical forces were involved in this process. Within 24 hours, the CLS were formed. We observed by confocal microscopy that during the CLS formation the topography of the integrin αvβ3 was modified. A dot-point labelling on the cell surface was observed during the first hours of cell adhesion on the fibrin, while a diffuse labelling in all the cell body was observed when the CLS were formed. Furthermore, a FACS analysis of the αvβ3 expression on HUVECs during the in vitro angiogenesis showed that the expression of this integrin was down-regulated in time. Our hypothesis is that the mechanical forces, transmitted by the integrins to the cell, play a key role in the angiogenesis process. As it is known that αvβ3 is involved in the first steps of angiogenesis, this study raises the question to know whether the integrin αvβ3 intervene in the stabilization of the CLS formed.

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3-27

MAPPING OF THE COLCHICINE BINDING SITE ON TUBULIN BY KINETIC TECHNIQUES. E. Van Craenenbroeck, C. Dumortier and Y. Engelborghs

The polymerization of tubulin into microtubules is inhibited by the binding of colchicine to tubulin. To characterize the binding pathway and to map the binding site on tubulin, a series of kinetic studies have been carried out. The binding process was studied by observing the influence of structural changes in colchicine on the binding parameters. Competition experiments were done to characterize the binding site of colchicine on tubulin. The techniques used were fluorescence stopped flow and spectrofluorimetry.

This abstract deals with the following aspects of the study:

Colchicine consists of a trimethoxybenzene ring (A), a tropolone ring (C) and a double bridge between both (B). To determine the influence of the B-ring on binding, a colchicine derivative was studied which lacks a side group on the B-bridge (desacetamidocolchicine). Kinetic and thermodynamic properties of the binding were changed compared to colchicine, which indicates that the B-ring not only acts as a steric barrier, but also interacts specifically with tubulin.

The binding characteristics of 2',3',4'-trimethoxyacetophenone (TMA'), an A-ring analogue, were also studied and compared to earlier measurements on 3',4',5'-trimethoxyacetophenone (TMA). Both substrates show thermoneutral association kinetics, with TMA' having the lowest affinity for tubulin. This indicates that the position of the methoxygroups on the A-ring are of steric importance to the binding.

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3-28

SIMULTANEOUS DETECTION OF CELL-CELL AND CELL-SUBSTRATE-INTERACTIONS. J. Wegener, A. Janshoff, J. Seebach, M. Sieber and H.-J. Galla

Experimental methods to monitor the properties of living cells in tissue culture in real time are rather rare and usually of qualitative nature. We developed an experimental approach based on gold surfaces as culture substrates, that enables to study cell adhesion as well as formation and modulation of epithelial cell-cell-contacts (tight junctions) in real time.

The core component of the experimental setup is an AT-cut quartz resonator with a fundamental resonance frequency of 5 MHz, which is on both sides covered with thin gold electrodes. Impedance analysis of the quartz resonator in a frequency range between 4.97 and 5.04 MHz and subsequent equivalent circuit modelling allows to deduce specific parameters that describe the shear oscillation of the quartz. Adhesion of animal cells onto the top surface of the quartz resonators alters the characteristics of its shear vibration which are accessible by impedance analysis. The observed alterations are mainly due to cell-substrate interactions. In a second measuring mode impedance analysis of the cell covered top electrode in combination with an external counter electrode (1 Hz - 1 MHz) allows to quantify the passive electrical properties of the cell layer under investigation.

The combination of both measuring modes allows to investigate cell-substrate and cell-cell-interactions within one experiment. We used this powerful technique to study the time course of cell adhesion onto the quartz surface and the establishment of tight junctions. We could document the delay and finally the suppression of cell adhesion by increasing concentrations of the RGD-tripeptide within the culture medium. Perturbation of the actin cytoskeleton by cytochalasin D and the corresponding changes in cell-cell and cell-substrate interactions of confluent cell layers could be followed with good time resolution. We furthermore investigated the influence of non-isotonic media on cellular parameters.

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3-29

MARCKS-RELATED PROTEIN (MRP) ACCELERATES ACTIN POLYMERIZATION. F. Wohnsland, M. Steinmetz, U. Aebi, and G. Vergères

Actin is a 42 kDa protein which is - due to its ability to polymerize into filaments (F-actin) - one of the major constituents of the cytoskeleton. In 1992, Hartwig and Aderem (Nature 356; 618-622) proposed that MARCKS protein (Myristoylated Alanine Rich C Kinase Substrate) plays an important role in regulating the structure of the cytoskeleton by cross-linking actin filaments.

We investigated the effect of MRP (MARCKS-Related Protein), a 20 kDa member of the MARCKS family, expressed mainly in brain and reproductive tissues, on both the kinetics of actin polymerization and on filament structure. The influence of MRP on the kinetics of polymerization was tested by observing the fluorescence change of pyrene-labeled actin. We could show that MRP accelerates actin polymerization. The influence of MRP on the structure of F-actin was obtained by electron microscopy and viscosimetry. The results from both methods indicate a bundling activity of MRP. Since ultracentrifugation experiments show that MRP forms dimers, we propose that this dimerization process is necessary for the cross-linking and/or bundling of actin.

Interestingly, the activity of MRP on actin was retained, if not increased, in the presence of lipid vesicles. This result suggests that MRP might actively regulate the dynamics of actin polymerization at membrane surfaces.

Finally, to investigate the properties of the MRP effector domain, we have synthesized a 25 amino acid residue long peptide. Using the assays described above, we showed that the peptide alone is capable of inducing formation as well as strong bundling of F-actin filaments.

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4-1

FUNCTIONAL LIMITS TO IDENTIFY THE OUTER HAIR CELL MOTOR. J.E.Gale*, G.Géléoc and J F Ashmore.

Two distinct hair cell types are found in the mammalian cochlea. Both types are mechanotransducers responding to deflection of their apical stereocilia and are organised in a relatively invariant configuration along the cochlear length. Inner hair cells form the end organs of the auditory nerve, acting like 'conventional' hair cells. Outer hair cells in addition generate axial forces at frequencies up to at least 20kHz and are considered to be responsible for augmenting cochlear mechanics. The molecular basis for reverse transduction in outer hair cells is not known. The presumed motor protein occupies more than 40% of the basolateral membrane and it must undergo an area change of about 4% as the membrane potential changes from -150 to +100mV. The area change is associated with a charge movement which, measured in whole cell and in single patches, is equivalent to approximately 1 e^- being transferred across the membrane. Single patches 2.5 μm in diameter exhibit a charge transient with relaxation time constants down to 8.4 μs at 20°C. We find that the underlying kinetics is voltage-dependent. Associated with the charge movement, the effective voltage-dependent membrane capacitance can be measured at frequencies up 30kHz and provides a consistent estimate of the relaxation time constant. Although several molecular candidates for the motor molecule have been proposed, the present data suggest that a modified ion channel or an anion exchanger is unlikely and we have been exploring models of the motor which more closely resemble cation exchangers. *(Supported by the Wellcome Trust, Defeating Deafness and the Royal Society.)*

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4-2

STRUCTURE AND MECHANISM OF SELF-ASSEMBLING TRANSMEMBRANE CHANNELS. Eric Gouaux.

α-Hemolysin (αHL) from *Staphylococcus aureus* is a member of a family of toxins that are active against erythrocytes and leukocytes and that includes the γ-hemolysins and leukocidins, also from *S. aureus*. αHL self-assembles from a 33.2 kDa water-soluble monomer to a membrane-bound heptamer on the surface of erythrocytes and forms lytic transmembrane channels. Although the sequence identity between αHL and the other family members is relatively low (≤27%), inspection of the recently solved αHL heptamer structure (Song *et al.*, Science **274** 1859-1866 (1996)) and localization of the 28 strictly conserved residues supports the conclusion that the γ-hemolysins and leukocidins have three-dimensional structures similar to αHL. In terms of function, αHL is related to aerolysin from *Aeromonas hydrophila* and protective antigen from *Bacillus anthracis*, other heptameric channel-forming proteins, although there is no significant amino acid sequence identity. Since the structure of the membrane-bound form of αHL has been determined to 1.9 Å resolution, it is a paradigm for the structure of heptameric transmembrane channels formed by 14-strand antiparallel β-barrels. In addition, the αHL heptamer structure provides insight into protomer-protomer interactions, heptamer-membrane interactions, transport properties of the channel and mechanisms of assembly.

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4-3

AQUAPORINS AND CLC CHANNELS: TRANSPORT OF WATER AND ANIONS ACROSS PLANT CELLULAR MEMBRANES.

C. Maurel, C. Lurin, J. Güclü, D. Geelen, P. Gerbeau, H. Barbier-Brygoo, J. Guern, F. Tacnet* and P. Ripoche*

Aquaporins have been identified in the plasma membrane (PM) and vacuolar membrane (tonoplast; TP) of plant cells. Expression of these proteins in *Xenopus* oocytes indicated that they function as water-selective channels. Using stopped-flow spectrophotometry, we now show that TP vesicles purified from tobacco cells are 100-fold more permeable to water than their PM counterparts. The activation energy and sensitivity to mercury ions of water transport point to different mechanisms of permeability in the two membranes, by diffusion of water molecules across the lipid matrix in the PM and across water-channels in the TP. These findings suggest a role for water channels in the osmoregulation of plant cells, directly related to their typical compartmentation in cytoplasm and vacuole. Plant ion channels, and in particular anion channels, play an important role in cell osmoregulation and signalling. We have recently identified cDNA clones from tobacco and *Arabidopsis thaliana* that encode plant homologues of the animal CLC voltage-dependent chloride channels and induce anion currents when expressed in *Xenopus* oocytes. The function and regulation of this novel type of plant ion channels are investigated by a combination of molecular and genetical approaches.

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4-4

MECHANISMS OF GATING IN CLC CHLORIDE CHANNELS. M.Pusch, U. Ludewig, K. Steinmeyer & T. J. Jentsch.

CIC proteins are a gene family of voltage-gated Cl^- channels with two members involved in human diseases (CIC-1 and CIC-5). We analysed CIC-channels after expression in oocytes with electrophysiological techniques. Recently, we and others (Ludewig et al., Nature 383:340; Middleton et al., Nature 383:337) have shown that the *Torpedo* channel, CIC-0, is a dimeric "double-barrelled" channel with two identical protopores that gate independently from each other (fast gate) as originally proposed by Miller (1982). The slow gate that closes both protopores simultaneously depends on the properties of both subunits. We found that fast gating of individual protopores is dependent on voltage and on $[Cl]_{ext}$ and $[Cl]_{int}$. Both depolarized voltage and high external permeant ion concentration increase p_{open}. Fast gating properties of CIC-0 can be explained by a model in which the permeant anion serves as the gating charge, i.e. voltage-dependent binding of external Cl^- leads to an opening of the channel (Pusch et al., Nature 373:527). $[Cl]_{int}$ stabilizes the open state (Ludewig et al., J. Pysiol. 498:691). Additional support for the extrinsic gating mechanism came from the properties of a CIC-0 point mutant (K519E). Analysis of several mutants in the region surrounding K519 (highly conserved between CIC-0, CIC-1, CIC-2 and CIC-K) revealed effects on permeation and gating of single protopores, and on slow gating. We propose that the region around K519 contributes to the inner mouth of the ion conducting pore. Insights into the gating-mechanism of the muscular CIC-1 came from mutations that cause dominant myotonia. Several point mutations found in affected families (G200R, I290M, R317Q, P480L, Q552R) drastically shift steady-state p_{open} to more positive voltages in mutant/WT heterooligomeric channels and even more so in mutant homooligomers, fully explaining why they cause dominant myotonia. These mutants probably affect a gating mechanism that is common to both protopores of the presumably double-barrelled CIC-1, suggesting a stronger coupling of the gating mechanisms in CIC-1.

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4-5

TOPOLOGIES OF α-HELICAL PEPTIDES IN LIPID BILAYERS BY SOLID-STATE NMR SPECTROSCOPY, B. Bechinger

Hydrophobic or amphiapthic α-helical polypeptides like alamethicin, melittin (Z=5), magainins (Z≥4), signal sequences, fusogenic or model peptides have been shown to cause membrane disruption and/or the increase in bilayer permeability. Previous investigations indicate that highly charged channel-forming polypeptides exhibit an orientation parallel to the membrane surface, whereas other more hydrophobic polypeptides adopt a transmembrane alignment. The structures of the single channel-forming structures are very difficult to determine, however, as only a minor fraction of the polypeptides may be responsible for the electrophysiological and cell killing properties measured.

In order to better understand the interactions that determine the orientation of membrane-associated polypeptides a series of hydrophobic model peptides containing histidines was prepared by solid-phase peptide synthesis. The orientation of these peptides was determined in uniaxially oriented lipid bilayers by proton decoupled ^{15}N solid-state NMR spectroscopy. pH changes cause some of these α-helical peptides to flipp from orientations parallel to the membrane surface to transmembrane aligments indicating that the modulation of the charge distribution as well as hydrophobic interactions are important factors of membrane topology. The experimental results permit to check the validity of theoretical considerations concerning the energetics of protein-lipid interactions. They also provide a theoretical background which allows to analyse the NMR-structures and membrane alignments of signal sequences and protein channel fragements as well as to evaluate current models for channel formation.

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4-6

EFFECTS OF NEUTRALIZATION OF GLUTAMATE RESIDUE 340 ON DIVALENT CATION BLOCKAGE IN THE CYCLIC NUCLEOTIDE-GATED CHANNEL CLONED FROM BOVINE OLFACTORY RECEPTOR NEURONS. P. Gavazzo[1], C. Picco[1], E. Eismann[2], U.B. Kaupp[2] and A. Menini[1].

Ion channels directly gated by cyclic nucleotides (CNG channels) are present in the plasma membrane of photoreceptors and olfactory receptor neurons and in a variety of other cells. When CNG channels are open they conduct a cationic current and do not appreciably select between different monovalent cations; divalent cations also permeate the channel but at the same time they block the current carried by monovalent cations in a voltage- dependent manner. It has been shown that in the bovine retinal rod channel a glutamate residue, Glu363, is within the putative pore region and controls the blockage of the channel by external divalent cations. In this work, we studied the role of the equivalent residue, Glu340, in the bovine olfactory CNG channel. Point mutations were introduced in the wild type cDNA of the channel: Glu340 was substituted with different non charged amino acids such as Gln, Asn, Thr, Gly. The wild type and mutant channels were expressed in Xenopus oocytes, and CNG currents were measured in patches excised from oocytes. Our results showed that neutralization of Glu340 residue reduced the external blockage of divalent cations. On the contrary, the internal blockage of divalent cations was not abolished by the substitution of Glu340, suggesting the existence of an additional divalent cation-binding site at intracellular surface of the pore.

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4-7

FUNCTIONAL CHARACTERIZATION OF A MEMBRANE PROTEIN WITH A DUAL FUNCTION OF TRANSPORTER AND CHANNEL. L. Plançon, M. Bonhivers, P. Boulanger, A. Ghazi, M. Le Maire and L.Letellier

The *E. coli* outer membrane protein FhuA (MM = 79 kDa) catalyzes the transport of Fe^{3+}-ferrichrome and is the receptor of phage T5. FhuA was purified chromatographically in octyl glucoside. The protein is monomeric and essentially organized in β sheet (51%). Functionality of the purified protein was assessed from fluorescence measurements using the DNA probe Yo-Pro-1. Interaction of nM concentrations of FhuA with phage T5 resulted in the release of almost all of the phage DNA (1). Binding of phage T5 to FhuA inserted into planar lipid bilayers resulted in appearance of high conductance ion channels. Analysis of the electrophysiological characteristics of the channels suggests that binding of phage T5 to one of the external loop (322-355) of FhuA, which constitutes the T5 binding site, unmasks an inner channel in the protein (2). FhuA was reconstituted into large unilamellar liposomes. Binding of phage T5 to FhuA caused the transfer of phage DNA inside the vesicles and an efflux of entrapped siderophores (3). Taken together these results suggest that both DNA and siderophores diffuse through the channel opened by the phage in FhuA. This probably constitutes the route used by phage DNA and siderophores to cross the *E.coli* outer membrane.

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2. Bonhivers et al., (1996), EMBO J., 15, 1850-1856
3. Letellier et al., (1996), J. Biol. Chem., in press

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4-8

K_{ATP} CHANNEL REGULATION BY INTRACELLULAR DIADENOSINE POLYPHOSPHATES. C. Ripoll, J.M. Rovira and B. Soria.

The implication of ATP-sensitive K^+-channel (K_{ATP}) in insulin secretion is well established. It is closed by glucose metabolism, which stimulates pancreatic β-cell secretion. Metabolic regulation is mediated by changes in intracellular ATP and ADP. We have recently reported that other nucleotides, diadenosine polyphosphates (DPs) are very effective inhibitors of K_{ATP} channels. We have shown that these compounds are found in β-cell cytosol, and their concentrations increase by several ten-fold in the presence of stimulatory glucose concentrations, reaching the micromolar range, in which they can effectively block channel activity. The aim of the current study was to provide some mechanistic evidence about DPs- K_{ATP} interaction, and how these compounds are involved in channel gating modulation, in comparison with ATP and ADP roles. For this purpose, single-channel currents were measured in the inside-out configuration of excised patches from cultured mouse β-cells. Application of DPs to the inner side of β-cell membranes markedly depressed channel activity, with a time course similar to that found for ATP. This effect was completely reversible after DPs wash-out. Contrariwise to ATP, DPs were not able to prevent the run-down of the channel open probability or to re-activate channel after preceding run-down. DPs blocking effect was not altered in the presence of ATP. Our results indicate that although DPs specifically inhibit K_{ATP} channel there are some differences in the mechanism of inhibition by DPs and ATP. It may thus be assumed that DPs may not act at the same binding sites as those for ATP in K_{ATP} channel .

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4-9

STEROL-DEPENDENT PORE FORMATION OF SYRINGOMYCIN E ON RED BLOOD CELL MEMBRANES. **G. Ágner, L.V. Schagina, Yu.A. Kaulin, J.Y. Takemoto, K. Blaskó**

The antifungal Syringomycin E (SRE) is a lipodepsipeptide produced by the phytopathogen Pseudomonas syringae pv. syringae. The plasma membrane has been found to be the primary target of its toxic activity.

In this work the pore formation activity of SRE in red blood cell membrane was evaluated by tracer kinetic and hemoglobin transport measurements. The toxin exposed to the RBCs above a threshold concentration of 2.10^6 molecules/cell causes a partial lysis of cells evolved and completed within 2 minutes and increases the membrane permeability of the lysis survived cells to ions. Both the lytic and permeability increasing effects of SRE shows similar linear concentration dependence. Above the concentration of $6\text{-}8.10^6$ molecules/cell the time course of ^{86}Rb efflux gives an exponential function approaching to the equilibrium tracer distribution. Below this concentration the transport kinetic curves get to saturation before reaching the equilibrium tracer distribution. Similar kinetic data were obtained for hemoglobin efflux. This observation may be explained by ionconducting SRE pore formation and then a time dependent inactivation of pores. The radius of pores was estimated by electric conductance measurements on model lipid bilayers and found to be about 1.5 nm. This size of pores means pathway for free diffusion of ions and hemoglobin monomers or dimers.

The sterol composition of RBCs was altered by liposome pretreatment. Depletion of cholesterol content of the RBC membrane or its partial substitution by ergosterol enhances the affinity of the membrane for SRE. The affinity was characterized by the permeability coefficient (p) for ^{86}Rb ion of the modified membrane. SRE exposed to the cells with inherent cholesterol content in a concentration of 6.10^6 molecules/cell modified all cells and $p = 1.10^{-7}$ cm/s was found. Cholesterol depletion by 50% or ergosterol substitution by 10% resulted a partial modification of RBCs but a 5 times higher permeability of the modified cells indicating higher affinity of cells for SRE.

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4-10

INTERCELLULAR COUPLING IN THE PANCREATIC ISLET OF LANGERHANS: MINIMAL NUMBER OF COUPLED β-CELLS NEEDED TO SHOW OSCILLATORY ELECTRICAL ACTIVITY. E.Andreu, R. Pomares, C. López-García, B.Soria & J.V. Sanchez-Andrés.

Pancreatic islets of Langerhans, consist of some thousand of β and non-β (α,δ,PP) cells clustered and coupled through gap-junctions. At intermediate glucose concentrations (7-20mM), β cell membrane potential oscillates with a typical square wave bursting pattern. This behavior is only observed with β-cells recorded from the intact islet as far as isolated cells are unable to oscillate. Since oscillatory activity seems to be an emergent property of coupled cells (J.Physiol. 498:753-761) it was interesting to examine how many cells are needed in order to make possible the oscillatory behavior. Methods: The β and non-β cell distribution in the islets of albino mice was studied by immunohistochemistry. Electrical coupling was studied by simultaneous recording from two neighbor β cells. Results: 1) β cells are coupled in small clusters interconnected all over the islet and surrounded by other cell populations (α,δ,PP), that are also coupled. 2) The values of coupling conductance during the oscillatory activity (≈200-500 pS), and of input resistance for that same period (≈100-200 MΩ), together with the input resistance of isolated β cells (some GΩ), makes possible to estimate the needed number of cells that have to be coupled in order to make the values of input resistance of the coupled cell to be in the optimal range for oscillatory behavior. 3) It is concluded that a small cluster of β cells (9-25 cells) should be enough to generate an oscillatory activity provided that coupling conductance oscillates between two levels

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4-11

A6 CELLS APICAL MEMBRANES PERMEABILITY TO CHLORIDE IONS IS INCREASED BY LUMINAL ATP VIA A RISE IN THE NUMBER OF ACTIVE CHANNELS. U. Banderali, E. Brochiero and J. Ehrenfeld.

A6 cultured epithelial cells derive from *Xenopus Laevis* kidney and represent an excellent model of salt transporting tight epithelia. In A6 epithelia, chloride secretion can be stimulated by several extracellular factors. In the present study, the stimulation of chloride secretion by luminal ATP is addressed. Measurements of short circuit currents, experiments in spectrofluorimetry and isotope fluxes showed that addition of 0.3 mM ATP to the luminal fluid increases the permeability of apical membranes to chloride ions. We used the single-channel patch-clamp technique to characterize the channels involved in this process. We showed that addition of ATP to the luminal solution stimulated chloride channels activity in 23% of successful patches on the apical membranes of the epithelium. The single-channel conductance of these channels was 7±2 pS. In 64% of cases stimulation induced the activation of one or more channels in initially silent patches. In the other cases, activity of such 7 pS channels was already observed in control conditions and stimulation by luminal ATP increased the value of $N*P_o$ (N=number of simultaneously active channels, P_o=single-channel open probability). According to statistical analysis of the single-channel recordings *(Denicourt et al. 1996, Biochim. Biophys. Acta, 1285:155-166)*, these results were consistent with an increase in N and no significant change in P_o after addition of ATP. Our results indicate that the overall increase in permeability occurs primarily by turning on initially quiescent channels.

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4-12

PHAGE LAMBDA CONVERTS THE PORIN LAM B INTO A NOVEL HIGH CONDUCTANCE CHANNEL. Berrier C., Letellier L., Bonhivers M., and Ghazi A.

The *Escherichia coli* outer membrane porin LamB functions as a general water-filled channel, with a markedly specificity for maltodextrins. LamB is also the receptor for bacteriophage lambda. The purified protein was first reconstituted in liposomes and then inserted in planar lipid bilayers. Under these conditions, the channel presents the known electrophysiological behavior, previously described in the litterature (conductance, kinetic gating, selectivity...). Addition of phage lambda to proteoliposomes, prior to their fusion in lipid bilayers, resulted in the appearance of a high conductance ion channel whose electrophysiological characteristics were totally different from those of LamB. Furthermore this channel was never observed with phage lambda. These data suggest that the interaction of phage lambda with the porin LamB triggers a conformational change in the channel that results in an increase of LamB conductance. Recently, Bonhivers and al., 1996, EMBO J, 15 1850-1856, showed that the transporter FhuA is converted into a channel upon binding of phage T5. Conversion of bacterial outer membrane transporter/channel into high conductance channels upon binding of a phage appears a general rule. Such studies are of a general interest for the understanding of phage DNA transfert and of the mechanism of pore-gating of β barrels proteins.

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4-13

SODIUM TRANSPORT AND DISTRIBUTION IN SWEET PEPPER DURING AND AFTER SALT STRESS. M. Blom-Zandstra.

Sodium uptake and distribution in salt-stressed sweet pepper plants are strictly regulated. Sodium transfer by the xylem is mainly directed to the (basal) pith cells of the stem, while no Na+ is carried off to the phloem. Plants transferred to a NaCl-free solution, showed a release of Na+ from the pith cells to the the roots and outward the plants. In patch clamp (WC) experiments, the plasma membrane (PM) of unstressed pith cells contained only outward rectifying currents (ORC's), highly selective for K+. After a stress period (15 mM NaCl), the ORC's were also selective for Na+, indicating that Na+ stress changes the PM transport characteristics.

To study the changes, we evaluated the Na+ distribution during and after NaCl stress by 1. xylem sap analyses and 2. by 22Na-pulse-labelling experiments in a split-root sytem, partly feed with 22Na+. Xylem sap analyses showed a strong decrease of the [Na+] from stem basis to top during NaCl-stress, indicating a high Na+ absorbance capacity of the basal pith cells. After NaCl-stress, the [Na+] decreased and the gradient along the stem disappeared. The 22Na+ experiments showed that no Na+ was transferred from stem to roots during NaCl-stress, indicating that no Na+ is released to the phloem. However, after NaCl-stress, Na+ was transferred from stem to roots. Thus, changes of the PM characteristics during salt stress, as shown by the patch clamp experiments are only expressed in an actual release of Na+ from the pith cells and consequential change of the transport profile after transfer of the plants to a NaCl-free nutrient solution.

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4-14

ELECTROPHYSIOLOGICAL STUDIES ON THE ACTIVATION PATHWAYS OF MACROPHAGES BY ENDOTOXIN.

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We are investigating the physico-chemical characteristics underlying the interaction of endotoxin (lipopolysaccharides, LPS) with the cytoplasmic membrane of monocytes / macrophages, finally leading to their stimulation to cytokine production.

Resonance energy transfer experiments using double labeled phospholipid (PL) liposomes showed that endotoxin monomers or small oligomeres intercalate into PL-matrices in the presence of the lipopolysaccharide binding protein (rLBP). LBP was thus shown to function as a transport protein for endotoxin but also for negatively charged PL.

The lipid anchor of LPS, lipid A, is known to represent the endotoxic principle of LPS. With synchrotron small angle X-ray diffraction we could determine the supramolecular structure of lipid A aggregates and from this deduce its molecular conformation. We found a correlation between the conformation of various lipid A and their capability to induce cytokine production.

Thus, two possible activation pathways may be discussed, (i) a binding of lipid A to the transmembrane signal transducing protein - possibly an ion channel - leading to its activation in dependence on the conformation of lipid A, and (ii) a change of the transmembrane potential as a consequence of the high negative charge density of the intercalated endotoxins.

We present first results from (i) potential measurements with planar lipid bilayers in the presence and absence of LPS / LBP demonstrating the intercalation of LPS, (ii) experiments on the direct stimulation of cells using the voltage clamp technique to control the membrane potential, and (iii) studies on the influence of LPS / LBP on the channel characteristics in macrophages.

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4-15

DOES *SACCHAROMYCES CEREVISIAE* HAVE AQUAPORINS?

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Analysis of the *S. cerevisiae* genome sequence revealed the presence of a putative water channel (aquaporin) (Park and Saier, J. Membrane Biol., 153, 171-180, 1996). Using PCR, we amplified this candidate water channel (*AQY1*) localized on chromosome XVI from *S. cerevisiae* genomic DNA. This codes for a 327 amino acid polypeptide (Aqy1p) with 6 predicted transmembrane domains. This sequence was subcloned and the cRNA was transcribed *in vitro*. Oocytes micro-injected with the *AQY1* cRNA and incubated between 4 to 5 days in isoosmotic media exhibited an increase in osmotic water permability when placed in hypoosmotic media. These results allowed us to identify the first known aquaporin gene in yeast. Sequences amplified from other strains of *S. cerevisiae* did not all display water channel activity, suggesting an evolutionary elimination of the water channel activity in laboratory strains.

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4-16

CHARACTERIZATION OF A SMALL CONDUCTANCE VOLTAGE INDEPENDENT Ca^{2+}-ACTIVATED K^+ CURRENT IN ISOLATED HIPPOCAMPAL CA1 NEURONS. J.A.Borgdorff, W.J.Wadman.

Ca^{2+}-activated K^+ currents play an important role in shaping the firing pattern of neurons. In hippocampal CA1 neurons two Ca^{2+}-activated K^+ currents have been identified, contributing to spike frequency adaptation and spike repolarization. We characterized a small conductance, voltage independent Ca^{2+}-activated K^+ current in freshly isolated CA1 neurons, using a combination of whole-cell patch clamp recording and Ca^{2+} ratio imaging. $[Ca^{2+}]i$ was manipulated with the Ca^{2+} ionophore ionomycin, creating a quasi-steady state during the recordings, and monitored with the fluorescent probe Fura-2. Current properties were analyzed under voltage clamp conditions. Fluctuation analysis provided insight in single channel properties.

The Ca^{2+}-activated K^+ current showed a high Ca^{2+} sensitivity that was well described with a Hill equation. Kd; 210±20 nM; Hill coefficient: 4.7±0.5 (means ± s.e.m.; n=8). Both parameters were independent of membrane potentials. Raising $[Ca^{2+}]i$ from 40±10 nM to 550±60 nM under physiological [K+] conditions, increased the membrane conductance at -85 mV from 0.44±0.12 to 3.3±0.46 nS, respectively (n=6). The current persisted during long exposure to high $[Ca^{2+}]i$. Fluctuation analysis indicated channels of 2-5 pS (n=7; $[K+]i=[K+]o$=140 mM), with [Ca2+]i acting through modulation of the channel open probability. For $[Ca^{2+}]i$ > 500 nM a second, voltage dependent Ca^{2+}-activated current was observed, most likely carried by BK channels. Current kinetics upon changes in $[Ca^{2+}]i$ remains to be elucidated.

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4-17

EFFECTS OF MONO- AND MULTI-VALENT CATIONS ON THE INWARD-RECTIFYING POTASSIUM CHANNEL IN PROTOPLASTS FROM MAIZE ROOTS. M. Bregante, A. Carpaneto and F. Gambale

Macroscopic voltage- and time-dependent currents mediated by potassium-selective inward channels were studied by the patch-clamp technique in protoplasts from cortical parenchyma cells of maize roots.These currents increased as a function of K^+ concentration displaying a very slow saturation with a Km=146 mM. Monovalent ions, like ammonium and rubidium, displayed very low permeability ratios, while sodium and lithium, did not permeate at all through the maize-root inward-channel. Submillimolar concentrations of Cs^+ were sufficient to block, in a voltage dependent manner, the inward currents, which were also reversibly inhibited by external submillimolar concentrations of the metal ions Ni^{2+}, Zn^{2+} and Co^{2+}. 1 mM La^{3+} only slightly decreased (≈10%) both the single channel conductance (9.2±1.2 pS in 100 mM potassium) and the macroscopic current. Contrary to Cs^+, inhibition induced by metal ions did not show any voltage dependence. These results suggest that, similarly to animal potassium channels, the inward channel of maize-root cortical cells has a narrow pore of permeation and metal ions decrease the K^+ current possibly acting on binding sites located outside the pore. We are currently analyzing the effects induced by the same divalents on KAT1 channel from *Arabidopsis thaliana* expressed in *Xenopus oocytes*.

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4-18

A ROLE OF CYTOSOLIC Ca-BINDING PROTEINS ON OSCILLATIONS OF CALCIUM CONCENTRATION. M. Marhl, M. Brumen, S. Schuster and R Heinrich.

Oscillations of the cytosolic calcium concentration have been intensively investigated, and although many partial mechanisms have been established their interrelations and their integrated role in this phenomenon are not yet fully understood. The present mathematical model gives an insight into a possible role of cytosolic Ca-binding proteins on calcium oscillations. It takes into account an internal calcium store, *e.g.* endoplasmic reticulum (ER). The ER membrane channels are assumed to operate according to the calcium-induced calcium release mechanism (CICR). Calcium is transported into the ER by the ATP-dependent active transport. Calcium leak flux from ER is taken to be dependent on electric potential difference across the ER membrane. Additionally, exchange of monovalent anions and cations between the ER and the cytosol is also considered because these ions present a considerable portion in the balance of electric charge in the solution. In particular, a special attention is given to the binding of calcium to proteins in the cytosol. Different binding properties of calcium with respect to the binding constants as well as the total protein concentration are considered. Their effect on frequency and amplitude of calcium oscillations is thoroughly analysed. It is concluded that the Ca-binding proteins with fast kinetics are essential for understanding a low spike concentration of calcium in spite of a relative high efflux of calcium from the ER. Furthermore, with increasing concentration they reduces the frequency of oscillations.

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4-19

ION TRANSFER PROPERTIES OF A PRIMARY AMPHIPATHIC PEPTIDE. L. Chaloin[1], E. De[2], P. Charnet[1], P. Vidal[1], L. Beven[3] G. Molle[2] and F. Heitz[1].

We have built a synthetic peptide with the following sequence Ac-M-G-L-G-L-H-L-L-V-L[10]-A-A-A-L-Q-G-A-K-K-K[20]-K-K-K-R-K-V-Cya to be used as a drug carrier through a covalent linkage involving the C-terminal cysteamide. At concentrations greater than 1μM the free form of this peptide has been shown to be cytotoxic on mycoplasms and L-1210 cells. At similar concentration, when incorporated either in Xenopus ovocyte membranes or in planar lipid bilayers (BLM) this peptide led to discrete fluctuations of the transmembrane currents indictive of the presence of single channels with a conductance of 280 pS in NaCl 1M. and with long open times (1 sec). In both systems while pore formation, and presumably peptide incorporation, appears to be voltage dependent, the channel properties are essentially voltage independent. Peptide-induced channels are mainly cationic (cation/anion ratio = 5) with the following sequence K ≥ Na> Li = TEA > choline. It is worth noting that channel activity appears in BLM with peptide concentrations 7 orders of magnitude lower than in X. ovocytes, although the basic channel properties are essentially the same. We conclude that (1) cytotoxicity of the peptide occurs mainly through permeabilization of the plasma membrane by peptide-induced formation of cation channels, (2) these channels can be formed in synthetic membranes and thus without the participation of any endogenous receptor and (3) both systems are equally useful to study the biological properties of this type of peptide.

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4-20

PLANAR LIPID BILAYER INCORPORATION STUDIES OF TWO HOMOLOGOUS 18-MER PEPTAIBOLS WITH DIFFERENT C-TERMINAL AMINO ALCOHOLS. P. Cosette, D. Duval, S. Rebuffat, B. Bodo, G. Molle.

Peptaibols are linear 7- to 20-residue antibiotic peptides rich in α-aminoisobutyric acid (Aib) with a C-terminal amino alcohol and which usually adopt a predominant α-helical structure. They induce ion channels in planar lipid bilayers by aggregation of monomers, according to the barrel-stave model. In order to stress the influence of the C-terminal amino alcohol on the ion channel characteristics, we studied the voltage-dependent properties of two homologous 18-mer peptaibols, trichorzins PA V and PA IX, having tryptophanol (Trpol) and phenylalaninol (Pheol) as C-terminus, respectively. Both peptides were helical, as shown by CD and NMR data. Macroscopic conductance studies for PA V and PA IX displayed a similar voltage-dependent pattern, with an approximative Ve of 8-10 mV. Analyses of the I-V curves for different peptide concentrations allowed to estimate the mean number of monomers per conducting aggregate: it was smaller for the Trpol (5-7) than for the Pheol analogue (9-10). The single-channel formation analyzed at 15°C to slow down the kinetics, indicated faster current fluctuations for PA IX (0.5 ms vs 0.8 ms for PA V) and lower conductance levels for PA V, than for PA IX. These results pointed out the importance of the C-terminal Trpol side chain on the channel stabilization.

PA V: Ac Aib Ser Ala Iva Iva Gln Aib Val Aib Gly Leu Aib Pro Leu Aib Aib Gln Trpol
PA IX: Ac Aib Ser Ala Iva Iva Gln Aib Val Aib Gly Leu Aib Pro Leu Aib Aib Gln Pheol

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4-21

PERMEABILIZING ACTIVITY OF PHYTOTOXINS FROM *PSEUDOMONAS SYRINGAE* ON PURELY LIPIDIC MEMBRANES. M. Dalla Serra, P. Nordera, I. Bernhart, G. Fagiuoli, D. Di Giorgio, A. Ballio and G. Menestrina

Pseudomonas syringae pv. *syringae* is a phytopathogenic bacterium with a very wide range of hosts including agronomically important plant species. It produces two groups of lipodepsipeptides: the nonapeptides syringomycins (SR) and syringotoxins (ST), and the more recently characterised syringopeptins (SP) composed of 22 or 25 AA residues. Both of them contribute to a variety of bacterial plant diseases including necrosis of leaf and stone fruits and holcus spot disease of maize. They also inhibit the growth of several fungi and yeasts. We studied the activity of all four these lipodepsipeptides on purely lipidic membranes in order to get information on the binding and the permeabilisation. Both syringopeptins have a low specificity showing very high degree of binding and permeabilisation with all the tested lipid compositions. ST and SR instead have a higher activity if, respectively, lathosterol (which is mainly present in animal cell membranes) or ergosterol (mainly present in fungi membranes) are included as components in the lipid mixture. We then focused our attention on the pore-forming ability of SP_{25} added to an artificial planar bilayer system in a voltage-clamped configuration. We observed toxin channels with a complex voltage-sensitive behaviour: at negative voltages they open with a superimposed flickering of a low conductance state ($G \approx 10$ pS) and with a very short lifetime ($t \approx 0.1$ s); at positive voltages the low conductance state disappears and only channels with a high conductance and long lifetime are present ($G \approx 30$ pS and $t \approx 5$ s), which however tend to close. This behaviour is apparently independent of the lipid composition of the membrane. Furthermore, the number of channels formed in the membrane invariably decreases during the experiment.

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4-22

VOLUME REGULATIONS OF NEUROBLASTOMA CELLS.
LM. Dubois, B. Rouzaire-Dubois and S. Bostel.

Volumes of NG108-15 neuroblastoma cells were measured in order to characterize the mechanisms involved in volume regulation in isosmotic and anisosmotic conditions. Cell volumes were determined by electronic sizing. Membrane currents were recorded with the whole-cell patch-clamp technique. Five different volume regulating mechanisms were characterized.

1. Short term (minutes) Cl^--dependent volume regulation. The cells behave as perfect osmometers when tonicity was changed at constant Cl^- by adding sucrose or replacing NaCl by $CaCl_2$ or $MgCl_2$. In contrast, the volume was poorly dependent on tonicity when Cl^- was changed by adding NaCl or H_2O. This volume regulation was linearly related to the external Cl^- concentration.
2. Mean term (tens of minutes) Ca^{2+}-dependent shrinkage. Shrinkage was induced by cell stirring or after an hypotonicity-induced swelling. These volume decreases were abolished by caffeine but not by ryanodine or EGTA. Shrinkage was also induced by the Ca^{2+} ionophore ionomycin. This ionomycin-induced volume decrease was abolished by EGTA.
3. Mean term (tens of minutes) swelling- activated regulatory volume decrease. Cell swelling induced an outwardly rectifying Cl^- current which was blocked by the Cl^- channel blockers NPPB, DIDS and DIOA. When tonicity was reduced at constant Cl^- by replacing NaCl by $CaCl_2$ or $MgCl_2$, the volume increased and then slowly decreased towards is control vallue. This regulatory volume decrease was blocked by NPPB, DIDS and DIOA.
4. Long term (hours-days) NaCl-dependent shrinkage or swelling. Shrinkage was induced by the addition of H_2O to the culture medium. Swelling was induced by the addition of NaCl (but not sucrose) to the culture medium. These volume regulations were abolished by the protein synthesis inhibitor cycloheximide.
5. Long term (hours-days) K^+ channel blockers-induced swelling. Swelling was induced by the K^+ channel blockers TEA, 4-AP, Cs^+.

These results suggest that the volume of neuroblastoma cells is controlled by: background Cl^- or osmolyte channels (1); Ca^{2+}-activated Cl^- channels (2); swelling-activated Cl^- channels (3); NaCl-dependent synthesis of ion channels or transporters (4); K^+ channels (5).

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4-23

PANDINUS *IMPERATOR* SCORPION VENOM BLOCKS VOLTAGE-GATED POTASSIUM CHANNELS IN HUMAN LYMPHOCYTES
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Extracellular application of both Pandinus *imperator* whole venom and its peptide fraction Pi2 markedly reduced whole-cell K^+ currents of human peripheral blood lymphocytes with half blocking concentrations 10 ng/ml and 2 ng/ml, respectively. The K^+ channel block by the whole Pi venom and Pi2 was readily removable by perfusion of the cell with the control extracellular solution, an interesting result compared to the earlier observation of Pappone et al. (J. Neurosci. 7:3300 (1987)), who found that the crude Pi venom blocked voltage-gated potassium channels in nerve fibers at a much higher concentration and the effect was practically irreversible. These observations with Pi2 are in line with the results of F.Gomez-Lagunas et al. (J. Membrane Biol. 152:49 (1996)) on Shaker B K^+ channels. The effect of Pi2 on the kinetic parameters of the human lymphocyte potassium channels was further investigated. Activation and inactivation kinetics were both affected by Pi2, however, these changes were not as marked as the reduction of the current amplitudes. The voltage dependence of steady-state inactivation and the recovery from inactivation were not affected by Pi2. Flow cytometric measurements with the membrane potential sensitive oxonol dye revealed a depolarizing effect of Pi2 suggesting that it may influence human lymphocyte activation.

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4-24

TEMPERATURE ADAPTATION CHANGES ION CONCENTRATIONS IN SPERMATOZOA AND SEMINAL PLASMA OF COMMON CARP WITHOUT AFFECTING SPERM MOTILITY
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A study was made of the effects of hibernation on the spermation of common carp (*Cyprinus carpio L.*) and on the changes in the characteristics of the individual sperm cells. Measurements were carried out on semen samples from 10 warm-adapted and 10 cold-adapted animals.The sperm cells from the hibernated animals had a higher intracellular pH (7.4 ± 0.1) than that of those from the non-hibernated animals (7.1 ± 0.1). The pH of the seminal plasma of the hibernated animals (8.6 ± 0.2) was higher than that of the nonhibernated animals (8.3 ± 0.1). The concentration of spermatozoa in the ejaculates of hibernated animals was about half that for the nonhibernated animals ($0.7 \pm 0.1 \times 10^{10}$ vs $1.4 \pm 0.2 \times 10^{10}$ cells/ml). The Na^+ concentration of the seminal plasma of the hibernated animals (83 ± 12 mM) was higher, while the K^+ concentration (64 ± 11) was lower than the corresponding data for the warm-adapted animals (63 ± 10 mM and 87 ± 16 mM, respectively). In contrast, there was no significant difference between the intracellular free K^+ concentrations of the spermatozoa in the autumn and spring ejaculates (58 ± 8 mM vs 60 ± 7 mM). The ion compositions and concentrations of the blood sera of hibernated and nonhibernated animals were similar. A season-independent increase by 0.2 pH units occurred in the intracellular pH during motile phase of sperm cells activated by hypoosmotic shock. This increase could be blocked by the Na^+/H^+ exchange inhibitor amiloride in a concentration of 100 μM. The motile fraction and duration of motility of the spermatozoa from hibernated and nonhibernated animals exhibited similarities.

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4-25

AMINO ACIDS OF THE SEGMENT IVS6 HAVE MODULATORY ROLE IN THE STATE-DEPENDENT INHIBITION OF THE L-TYPE CALCIUM CHANNEL BY DIHYDROPYRIDINES. L. Lacinová, R. Kass, D. Triggle and F. Hofmann.

Dihydropyridine (DHP) calcium channel blockers are known to interact with both open and inactivated state of L-type Ca^{2+} channel with different affinities. We investigated the role of amino acids of the IVS6 segment of α_{1C} subunit in this interaction. The effect of three DHPs: (+)isradipine, DHPs with neutral and permanently charged site chain (Bangalore et al., Mol. Pharmacol. 46: 660-666, 1994) on wild type α_{1C} and Ch30 channels was tested. Ch30 channel (Schuster et al., EMBO J. 15: 2365-2370, 1996) had exchanged three amino acids in IVS6 of the DHP sensitive α_{1C} subunit for corresponding amino acids of the DHP insensitive α_{1E} subunit (Y1485I, M1486F, I1493L). The IC_{50}s for wild type and Ch30 channels were 16±2 nM and 1.7±0.3 μM ((+)isradipine), 270±17 nM and 2.5±1 μM (neutral DHP), 195±20 nM and 440±50 nM (permanently charged DHP), respectively. When the current was activated by long depolarizing pulse to the peak of current-voltage relationship, the block by (+)isradipine remained constant during the pulse in wild type channel, but was remarkable enhanced in Ch30 channel. Neutral DHP enhanced current decay in both wild type and Ch30 channels. In all cases the additional inhibition which developed during the depolarizing pulse in a drug presence had a monoexponential time course with time constants 46±7 ms (Ch30, (+)isradipine), 23±4 ms (Ch30, neutral DHP), 19±4 ms (wild type, neutral DHP). In contrast, permanently charged DHP did not affect the current inactivation during depolarizing pulse in both channels, neither was the IC_{50} changed by Ch30 mutations. We suggest that the amino acids in IVS6 segment modulate the high affinity inhibition of the inactivated state of the channel by neutral DHPs but do not affect the interaction with the permanently charged DHP.

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4-26

INSERTION OF BETICOLINS IN BLACK LIPID MEMBRANE AND ANALYSIS OF THEIR ELECTRICAL PROPERTIES. C. Goudet*, M. Ildefonse#, J-B. Thibaud* and M-L. Milat'.

Beticolins are yellow toxins produced by the phytopathogenic fungus *Cercospora beticola*. Twenty beticolins have been isolated. Using some of them, B1, B2, B0 and B13 we show that these toxins are able to form pores in artificial black lipid membranes after insertion. This technic allow to study and compare their electrical properties: In each case, pore formation seems to be magnesium-dependent. Currents observed are ohmics. Using different media (KCl, NaCl, LiCl and $CaCl_2$), we also show that these pores exhibit poor selectivity, carrying rather indifferently cations and anions. The aim of our study is to correlate *in fine* the spatial structure of the monomer of beticolin with the behaviour of the channel since, B2-B1 and B0-B13 are two couples of isomers, B2-B0 are ortho beticolins whereas B1-B13 and para-beticolins, two different types of cyclisation. Moreover, biophysical studies concerning the chelation constants of beticolins with divalent anions and the analyses of their polarity show that significant differences exist and these parameters could influence the organization of the beticolins into channel-like structures.

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4-27

POTASSIUM CHANNELS, PROLIFERATION AND CELL VOLUME. B. Rouzaire-Dubois and J.M. Dubois.

A variety of studies have suggested that K^+ channel activity is a key determinant for cell progession through G1 phase of mitosis (reviewed in 1-4). We have proposed the hypothesis that K^+ channels control the cell volume and the cytoplasmic concentration of a solute involved in DNA synthesis (2).

In order to test our hypothesis, we have measured NG108-15 cell volume and proliferation with and without K^+ channel blockers and after osmotic alterations of cell volume.

Mean cell volume and rate of cell proliferation were measured with a Coulter counter-channelizer.

The K^+ channel blockers TEA (1-10 mM), 4-AP (0.2-2 mM) and Cs^+ (2.5-10 mM) decreased the rate of cell proliferation and induced an increase in cell volume which reached a steady-state value after 24 h. The proliferation was fully inhibited when the volume was increased by 30% and 50% inhibition was achieved when the cell volume was increased by 11%.

In order to confirm a role for cell volume in proliferation, cell shrinkage and swelling were induced by osmotic alterations of the culture medium (see Dubois, Rouzaire-Dubois and Bostel, this session). When 65 mM NaCl was added to the culture medium, the cell volume increased by 25% within 2 days and the rate of cell proliferation was reduced to 18% of its control value.

When 30% H_2O was added to the culture medium, the cell volume decreased by 18% within 2 days. After this period of time, the rate of cell proliferation was increased by 32%.

From these results, we conclude that K^+ channels control mitogenesis via regulation of cell volume. The cell volume may play a role in cell growth by controlling the intracellular concentration of a solute favoring mitogenesis.

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4-28

GAP JUNCTION SIZE AND NUMBER AS DETERMINANTS OF OSCILLATORY ACTIVITY IN PANCREATIC ISLETS. B. Soria, E. Andreu, A.Charollais*, JV Sanchez-Andrés, F. Martin and P. Meda*.

It has been recently shown that coupling conductances between pancreatic islet β-cells determine the appearance of an oscillatory pattern (J.Physiol. Lond. 498: 753-761, 1997). Junctional conductances values should be kept within an optimal range in order to allow β-cells to act as an oscillator, a system which provides an oscillatory output to a continous non-oscillatory input (Lect. Notes Comp. Sci. 930: 85-89, 1995). We have tested whether does exist a correlation between the size and number of gap junctions and the oscillatory activity in distinct mammalian pancreatic islets (human, rat, mouse and pig). Methods: gap junction size and number was determined by semiquantitative analysis of freeze-fractured membranes. Oscillatory activity was monitored either by measurement of the membrane potential and/or by monitoring cytosolic calcium with fluorescent probes. Results: 1) Pancreatic islets from human cadaveric donors and mice (C57B1, NOD, NMRI and ob/ob) show moderate (human) and high (mouse) values in the size and number of gap junctions whereas rats (Sprague-Dawley, NEDH, Wistar) and pig have low values. 2) Membrane potential from mice pancreatic islet cells oscillate in the presence of stimulatory glucose concentrations. Both human and mice (NMRI) islets show glucose-induced oscillations in cytosolic calcium concentration, however rat and pig islets scarcely show this oscillatory activity. Conclusion: It is concluded that there exist a good correlation between gap junction density and oscillatory activity in pancreatic islets.

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4-29

ELECTRICAL AND MOTOR ACTIVITY OF VEINS IN LONG TIME OBSERVATION (UP TO 30 HOURS). **U. Welscher, D.G. Weiss, H. Danler-Oppl, E. Neu, W. Jaud and M.Ch. Michailov**

Portal vein is a classical preparation for physiological and pharmacological investigations. Previously we reported on immediate reactions to ions, anesthetics, and X-irradiation *(Welscher et al.: Eur J Physiol 430, R161, 1995; Arch Pharmacol 346, P46, 1992)*. Most biophysical and electrophysiological experiments with intracellular recording (ICR) last short time following adaptation. Specific experiments (concern. action mechanisms of radiogenic reactions in animal and human vasculature) need long time observations (> 10 h). In **guinea pig portal vein** (n=20) 60-90 min after addition of sucrose (5-10%, to prevent microelectrode dislocation) resting membrane potential (RMP; -58±4 mV, n=85) decreased (depolarization 10-20%) while burst (B) activity was irreversibly transformed into spike activity (amplitudes of AP, over 60 mV). **Rat portal vein** showed under the same conditions stable motor and electrical activity for more than 10 h (with ICR out of one and the same cell for more than 90 min). RMP was -55 to -58 mV, AP 60-65 mV, amplitudes of phasic contractions for both preparations were 1-2 mN (n=20). For more than 10 h the factors we tested had clear reproducible effects, e.g. **Ca^{++} decrease** (50% of normal: 2.5 mM) transformed B into spike activity / **Ca^{++} increase** (300%) prolonged B duration and interval, **adrenalin** (1 nM - 1 μM) increased conc. dep. frequency, but not duration, of both motor and electrical B activity, and **X-rays** induced a reversible tonic contraction without changes in el. act. (n=15) (possibly PIP_2/IP_3 and arachidonate effects). For selected experiments it is necessary to study effects of biophysical and physiological factors not only during short but also in long term observations (> 10 h) to take into account essential time dependent changes in basic physiological responsiveness.

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5-1

CONNECTION BETWEEN THE ACTIVITY OF PHOSPHOLIPASE A_2 AND THE STABILITY OF THE VESICLE SUSPENSION. T. Hønger and P. Høyrup.

It is widely found that the phospholipase A_2 (PLA_2) catalysed hydrolysis of phospholipids to lyso-phospholipids and free fatty acids exhibits a lag phase of slow hydrolysis succeeded by a phase of rapid turn over of the substrate. The origin of this characteristic reaction time course is, however, still not well understood. Using a snake venom PLA_2 and one-component 100 nm vesicles of phosphatidylcholines with varying acyl chain length and degree of saturation, we study the changes in lipid composition and morphology as a function of temperature during the hydrolysis time course by HPLC and pH-stat titration combined with static light scattering. We find a correlation between the structural stability of the vesicle suspension and the degree of hydrolysis. This observation is consistent with our previous ^{13}C and ^{31}P NMR studies of the PLA_2 hydrolysis time course. Our results imply that the transition from the lag phase to the region of extensive hydrolysis involves a disruption of the initial vesicle structure. The present work suggests that the stability of the vesicle suspension to PLA_2 hydrolysis may be related to the lipid dependent perturbations of the thermo-mechanical properties of the system.

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5-2

ELECTROSTATIC BINDING OF DNA TO CATIONIC LIPID MEMBRANES. J.O. Rädler

Cationic liposomes mixed with DNA rapidly fuse into dense liquid crystalline lipid-DNA condensates due to their strong electrostatic attraction. Such aggregates are currently considered as potential non-viral gene carriers in human gene therapy. In this context the physics of DNA-membrane interaction is crucial for the understanding of self-assembled lipid-DNA constructs. The structure of such aggregates consists of alternating layers of DNA and cationic membranes composed of charged as well as uncharged lipids. Thereby the 2-dimensionally confined DNA exhibits a regular interhelical packing distance dependent on the charge density of the binary lipid membrane. We investigated in particular the interaction of DMPC/DMTAP (dimyristoylphosphatidyl-cholin / dimyristoyl-trimethyl-ammonium propane) and 20bp doublestranded-oligonucleotides by X-ray diffraction, DSC and fluorescence spectroscopy. The oligo-DMPC/DMTAP condensates exhibit smectic A multi-lamellar order on the molecular level while the mesoscopic morphology resembles a fine mesh, as observed by negative staining EM. A positional DNA-DNA correlation can not be seen for short oligo nucleotides in contrast to lipid-DNA condensates with longer e.g. (48kbp) λ-DNA. As a function of temperature the lipid chain melting and the DNA denaturing transitions were observed. We also studied the adsorption of DNA and oligonucleotides to substrate supported cationic bilayers.

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5-3

Theoretical Analysis of Structure and Thermodynamics of Energy-Transducing Membrane Proteins.

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Computer simulation techniques are used to analyse functional mechanisms of the light-driven proton pump protein, bacteriorhodopsin, and the photosynthetic reaction centre. Quantum chemistry calculations have revealed possible water-retinal interactions in the active site of bR [1]. The thermodynamic stability of water molecules in a 'hydrophobic' region of the protein, that might form part of the proton transfer wire, will be discussed [2]. Free energy profiles along selected segments of the photocycle are presented (see accompanying poster).

A homology model has been constructed of the photosynthetic reaction centre from *Rhodopseudomonas capsulatus* [3]. This model has been used to rationalise the absence of binding of the cofactor bacteriopheophytin to a partially-symmetrized engineered mutant of this protein [4].

References.

1. M. Nina, B. Roux & J.C. Smith. Theoretical Analysis of Retinal Hydrogen-Bonding in Bacteriorhodopsin. *Biophys. J.* **68** 25-39 (1995).

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3. N. Foloppe, M. Ferrand, J. Breton & J.C. Smith. Structural model of the photosynthetic reaction centre of *Rhodobacter capsulatus*. *Proteins: Struct. Funct. Genetics.* **22** 226-244 (1995)

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5-4

REGULATION OF BILAYER PROPERTIES BY LIPID ENZYMES IN *ACHOLEPLASMA LAIDLAWII*. S. Berg, O. Karlsson, L. Li, S. Vikström and Å. Wieslander.

In the single membrane of the small bacterium *Acholeplasma laidlawii* (i) a constant surface charge density, (ii) similar phase equilibria, close to a potential bilayer-nonbilayer transition, and (iii) a nearly constant spontaneous curvature, are metabolically maintained for the lipid bilayer. A liquid-crystalline state is essential, but not actively regulated. Hence, the average acyl chain length ("lipid thickness") and unsaturation, and the average chain order, of the lipid bilayer compatible with cell growth, may vary substantially but in a coordinated manner. Membrane phospholipids and glucolipids are consecutively made in two competing pathways from a common phosphatidic acid (PA) precursor. The integral enzyme (glucosyltransferase) for the major nonbilayer-prone lipid monoglucosyl-diacylglycerol (MGlcDAG) demands a critical fraction of negatively charged lipids for activity. This is coupled to a conformational change of the enzyme, as analyzed by proteolytic resistance. Chain length of the DAG substrate (from PA by a phosphatase), but not curvature or phase equilibria, is important for activity. The MGlcDAG synthase is probably a main site for the lipid surface charge regulation, balancing the two pathways. The following, single glucosylation of MGlcDAG to yield the major bilayer-forming lipid DGlcDAG only occurs in the presence of substantial amounts of an activator lipid, i.e. phosphatidylglycerol (PG) from the other, phospholipid pathway. *In vivo* these two lipids are coordinately synthesized. Amounts of PG needed *in vitro* depend on bilayer curvature and phase equilibria, i.e. the fractions and nonbilayer propensities of several additives tested. Variations in synthesis rates are sufficient for *in vivo* changes of MGlcDAG and DGlcDAG important for the curvature. Domain formation of the PG activator is a potential, second mechanism for regulating DGlcDAG synthesis.

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5-5

INFUENCE OF ANNEXIN V ON THE STRUCTURE AND DYNAMICS OF PHOSPHOLIPID BILAYERS : A FLUORESCENCE AND NMR STUDY. P. Demange[1], O. Saurel[1], L. Cezanne[1], A. Milon[1], R. Huber[2], and J.-F.Tocanne .

The annexin V is a cytosolic protein which exhibits a voltage gated calcium channel activity *in vitro*. Its structure has been resolved at 2.0 Å resolution. Punctual mutants of the recombinant annexin V characterized by biophysical (X-ray diffraction, electron microscopy) and electophysiological (patch clamp) methods allowed us to define the part of this protein which plays a role of voltage sensor and ion selectivity filter. But the main question still remains : how does annexin V, a soluble protein, can translocate calcium ions across a lipid bilayer?

Fluorescence polarization and solid state NMR spectroscopy (^{31}P and ^{2}H) on phospholipid vesicles showed no modification of the conformational mobility of PC and PS acyl chains and of the orientation of PC polar head groups. The only modification observed was a decrease of the transverse relaxation time (T2), indicative of the perturbation of slow motions of phospholipid bilayers. In contrast, FRAP experiments on planar supported bilayer composed of egg-PC/brain-PS (90/10) with NBD-PS, NBD-PC or FITC-annexin V indicate that binding of annexin V to the lipids induces, in a dose dependent manner and in a reversible way, a decrease of the lateral diffusion of phospholipids until a complete immobilization of NBD-PS and annexin V. These results are in agreement with the Karshikov's model which suppose a peripheral interaction of annexin V with polar head groups of acidic phospholipids.

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5-6

THE SPONTANEOUS CURVATURE OF MEMBRANES.

H.-G. Döbereiner, O. Selchow.

We present a technique for the measurement of the effective spontaneous curvature of fluid lipid membranes. This quantity is – besides the elastic moduli – one of the fundamental parameters characterizing the bending elasticity of fluid membranes. Nevertheless, to our knowledge, it has never before been quantitatively measured for free vesicles. We will show that the tendency of a closed membrane to bend in mechanical equilibrium has two different physical origins. First, the intrinsic spontaneous curvatures of the monolayers combine into a local spontaneous curvature of the membrane, which deviates from zero whenever there is any asymmetry between the two sides of the membrane, e.g., different lipid composition or different adjacent solutions. Second, the difference in the number of molecules between two monolayers of a closed vesicle, i.e., the area difference between the inner and the outer monolayer, couples to the integrated mean curvature and gives rise to a non-local source of bending. These two effects can be described with an effective spontaneous curvature. We measure this quantity by comparison of the experimental mean shape obtained via video phase-contrast microscopy with the theoretical shape predicted by the area-difference-elasticity model. The method is quite general and allows to examine the spontaneous curvature induced by (almost) any kind of asymmetry across a membrane. We present results on uni-lamellar phosphatidylcholine vesicles with a typical radius of 10 μm in raffinose:glucose solution. Vesicles are swollen in a 75 mOsmolal raffinose solution and incubated in an iso-osmolal raffinose:glucose solution of varying glucose content. We find that even with symmetric sugar solutions each vesicle is characterized by a *different* effective spontaneous curvature corresponding to the varying area difference between the monolayers. In contrast, for all vesicles studied, there is an increase of the effective spontaneous curvature with glucose content of the outer solution which is approximately linear with an *universal* slope.

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5-7

LYSIS INDUCED BY (LEU, LYS) AMPHIPATHIC PEPTIDES: HYDROPHOBICITY, AND NOT THE PEPTIDE LENGTH, IS A CRITICAL PARAMETER FOR ACTIVITY.

S. Castano, I. Cornut, K. Büttner & J. Dufourcq.

The adequate design of Leu and Lys residues to generate highly amphipathic α helices with more than 15 residues proved to generate peptides as potent as the more active natural cytotoxins.

The decrease in length form 15 down to 5 residues shows that LD_{50} towards human erythrocytes increases very significantly and parallels the decreased efficiency to induce leakage from liposomes. Parallely the affinity of the peptides for lipids decrease with their shortening: hydrophobicity is the limiting parameter for binding to the membranes. However when bound the very short peptides are still intrinsically very active. Fluorescence of DNS and Trp used to label the Nterm and Cterm respectively indicate a burying in the bilayer limited to the lipid ester area. Fluorescence polarization measurements in solution show that the shorter peptides are monomeric while the longer ones (n≥15aa) form large oligomers. FET between Trp and DNS indicate that when bound to lipids oligomers dissociate.

Therefore it is proposed that the critical step for lysis is the invasion of the outer leaflet of membranes by positively charged peptides and is not due to channel like activity through organized α-helical bundles.

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5-8

STRUCTURAL DESCRIPTIONS OF LIGANDS IN THE BINDING SITE OF INTEGRAL MEMBRANE PROTEINS AT NEAR PHYSIOLOGICAL CONDITIONS.

A. Watts

Small molecules, such as hormones, neurotransmitters and drugs, induce receptor conformational changes and then concomitant profound cellular changes through their interactions with membrane bound proteins. In addition, nutrient transport into cells and toxin efflux occurs through specific proteins. Despite the importance of defining the structure and dynamics of ligand interactions with integral membrane proteins, atomic details of membrane protein structure and the nature of their interactions with ligands, are scarce. This scarcity is, in part, a result of the serious technical difficulties in routinely growing 2D or 3D crystals for diffraction studies, and because high resolution solution state NMR spectra of membrane-embedded proteins are broadened by anisotropic magnetic interactions resulting in the loss of the spectral resolution required for obtaining structural details. Thus, to date, no pharmaceutically important ligand has yet been defined structurally using difference electron density maps in any membrane bound target. Using solid state NMR approaches, it is now possible to define the structure of a small, isotopically (^{2}H, ^{13}C, ^{19}F, ^{15}N) labelled ligand in its binding site of a membrane bound protein, such as a receptor, solute transporter or ion ATPase, at near physiological conditions, that is, not frozen, fully hydrated and in natural membrane fragments. Using novel magic angle spinning (MAS) NMR methods, dipolar couplings can be reintroduced into the spectrum of labelled ligands in their binding sites of integral, fully functional membrane proteins to give interatomic distances to high precision (± 0.5 Å), as well as chemical shifts measured directly, to help in providing structural details for a bound ligand, as shown for analogues of drugs (imidazo-pyridines) used in peptic ulcer treatment in the gastric ATPase, and for acetyl choline in the acetyl choline receptor.

An indication of the potential of these new methods for resolving structural details about well-defined parts of important, and often pharmacological membrane-bound target proteins under near physiological conditions, will be given.

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5-9

INTERACTIONS OF HAV-VP3(110-121) SYNTHETIC ANTIGEN WITH NEUTRAL AND CHARGED LIPID MONOLAYERS

C. Mestres*, P. Sospedra*, F. Reig, I. Haro**and M.A. Alsina***

It is known that the entrapment of synthetic Hepatitis A virus (HAV) antigens into liposomes increases the immunoresponse that otherwise sometimes is poor (1).

In order to select the best lipidic composition of liposomes we have studied the interactions between the HAV synthetic antigen VP3(110-121) and neutral (DPPC), positively charged (SA and DPPC/5% SA) and negatively charged (DPPG and DPPC/5% DPPG) monolayers.

Compression isotherms of the different lipidic composition monolayers on peptide containing subphases were performed studying the effect of the peptide on the miscibility of the mixed monolayers. Moreover, kinetics at constant area of the monolayers above described on subphases where the peptide was injected were carried out. Superficial activity of the peptide was also assayed before the monolayer studies.

Although interactions were not strong, maximum values were found for positively charged monolayers followed by the neutral ones. Negatively charged monolayers showed the lower degree of interaction. These results are in agreement with the net negative charge of VP3 peptide.

(1) Just, M. et al, Vaccine 1992, 10, 737

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5-10

STABILITY OF CUPOLA-SHAPED BILAYER LIPID MEMBRANES AT THE PHASE TRANSITION OF LIPID.

V.Antonov,E.Shevchenko,E.Smirnova,N.Bogatyreva, A.Chernysh

Stable bilayer lipid membranes with mobile Plateau-Gibbs border have been formed .The precondition of the formation was the presence of a lipid coverage on the teflon surface near the hole,where the membrane has been formed.This allowed the movement of the border along the teflon surface after transformation of the planar bilayer into a cupola-shaped by bowing of the bilayer due to excess hydrostatic pressure.To evaluate the bilayer area and electric conductance the cyclic current-voltage curves have been registered.As the result the giant bilayers were obtained with an areas up to two orders larger the planar bilayer.Changes in lipid bilayer area depend on the temperature of the phase transition of lipid.Cooling of the expanded bilayer was followed by a significant shrinkage of the bilayer below the main phase transition from liquid crystalline state to the gel. We suggest that the observed behavior of the bilayer lipid membrane may be of importance for the lung alveolar dynamics.

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5-11

BINDING OF MARCKS-PSD AND MRP-PSD TO BLACK LIPID MEMBRANES AND MONOLAYERS.

G. Bähr, A. Diederich, G. Vergères, and M. Winterhalter

We used the method of Inner Field Compensation (IFC) for black lipid membranes and the Vibrating Plate technique (VP) for monolayers on a Langmuir trough to compare the binding behavior of basic peptides. We measured the binding of peptides corresponding to the PSD (**p**hosphorylation **s**ite **d**omain) of MARCKS (**M**yristoylated **A**lanine-rich **C** **K**inase **S**ubstrate) and MRP (**M**ARCKS-**r**elated **p**rotein), two proteins essential for brain development. The PSD of these proteins binds to membranes and to calmodulin, crosslinks actin filaments and is phosphorylated by PKC (**P**rotein **k**inase **C**). Since a serine residue in the middle of the PSD of MARCKS is replaced by a proline in MRP, it was investigated whether this change effects the binding behavior of the two peptides. To estimate the contribution of hydrophobic residues to binding we compared the PSD peptides with pentalysine, a reference substance with no hydrophobic groups. The KCl concentration in the electrolyte and the percentage of negatively-charged lipids in the membrane strongly affect the change of the transmembrane voltage upon binding of the peptides. We compare the results of our measurements on black lipid membranes and monolayers with results obtained with zeta potential measurements on liposomes published by Kim et al., *Biophys.J.* **67,** 227-237 (1994).

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5-12

PEG:2000-LIPID CONTAINING DPPC LIPOSOMES: A SPIN LABEL ESR STUDY.

S. Belsito, R. Bartucci and L. Sportelli.

Lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol) (PEG-lipids) are being developed for in vivo drug delivery. Using the spin label Electron Spin Resonance (ESR) technique we have studied the system composed of dipalmitoylphosphatidyl-choline (DPPC) suspensions containing different aumonts of dipalmitoylphosphatidyl-ethanolamine (DPPE) with attached PEG of molecular weight 2000. We found that bilayers are formed introducing up to 7-8 mol % of DPPE-PEG:2000 in the host DPPC matrix. Relative to the unmodified DPPC bilayers, the sterically stabilized liposomes have enhanced mobility in the interfacial zone and reduced chain mobility in the hydrocarbon region as well as reduced fluidity. A slight concentration-dependent increase of the main phase transition temperature and un upward shift of the pre-transition are also observed. With the incorporation of 10 - 40 mol % of DPPE-PEG:2000 two component ESR spectra with different motional properties are obtained indicating that bilayers and micelles coexist over this broad range of concentation of the PEG-lipid. Finally aggregates consisting of pure micelles are formed at higher concentration of the polymer-lipid.

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5-13

THE "PENETRATIN" PEPTIDE INTERACTING WITH PHOSPHO-LIPID BILAYERS. E. Bellet-Amalric, F. Graner, A. Renault, D. Blaudez and B. Desbat.

A peptide, namely the last 16 amino-acids of Antennapedia homeodomain, translocates across neural cell membranes without damaging them and binds to the cell nucleus [Derossi, D. et al. *Journal of Biological Chemistry* 269 10444-50 (1994); 271 18188-93 (1996)]. This involves crossing the hydrophobic barrier of phospholipid tails,a surprising achievement for a hydro-soluble peptide. Hypothetic explanations invoke an inverted micellar arrangement around the peptide. This question is still totally open, but it is nevertheless already commercialized as a vector coupled to intracellular or nuclear drugs, under the name "penetratin".

We have been studying this peptide using optical methods: surface infrared spectroscopy and ellipsometry. We demonstrated that the peptide actually interacts with phospholipids, mostly with phosphatidyl serine (PS), as expected based on their respective charges; and also with phosphocholines (PC). This interactions manifests itself through conformation and orientation changes of the peptide, and through orientation and pressure changes in the lipid layer. We are currently trying to resolve this interaction mechanism at the nanometer scale using neutrons, and the kinetics of this interaction using thermodynamical methods.

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5-14

RELATIONSHIP BETWEEN THE EXCESS OF PHOSPHOLIPIDS IN THE OUTER LEAFLET OF THE PLATELET PLASMA MEMBRANE AND THE CYTOSKELETAL REORGANISATION. N. Bettache, P. Mangeat and A. Bienvenüe.

The distribution of phospholipids between the two leaflets of the plasma membrane of many eukaryotic cells is asymmetric. This asymmetry is maintained in platelets as in erythrocytes by continuous transport of aminophospholipids (phosphatidylserine and phosphatidylethanolamine) from the outer to the inner leaflet by ATP-dependent aminophospholipid translocase, while the choline head phospholipids remain preferentially located in the outer leaflet. Addition of a short chain analogue of phosphatidylcholine in resting platelets induces a long-lasting shape change resulting in filopod formation while using an analogue of phosphatidylserine, platelets quickly return to their initial shape. Concomitantly to aminophospholipids exposure during Ca^{2+}-induced activation, filopod formation is also observed in platelets pre-treated with calpeptin (a permeant inhibitor of calpain). This shape change results in phospholipid excess in the outer leaflet of plasma membrane and in cytoskeleton reorganisation. In this study, we have investigated by immunofluorescence, the redistribution of actin-binding proteins (talin, filamin,....) in response to the addition of an analogue of phosphatidylcholine in the outer leaflet of plasma membrane of resting platelets or in activated platelets pre-treated with calpeptin. The actin-binding proteins are redistributed differently in the resting and the activated states of human platelets.

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5-15

PHOTOCURRENTS OF BACTERIORHODOPSIN DEPOSITED ON METAL ELECTRODE. Michel Déry and François Boucher

Bacteriorhodopsin is the light-activated proton pump contained in the purple membrane of some halobacteria.Charge displacements occuring during the different steps of its photochemical cycle generate characteristic currents. We report here a comparison between the directions and amplitudes of photocurrents in oriented (electrodeposited) and pseudo-random (dried) films of purple membrane deposited on platinum or tin oxide electrodes. Photocurrents measured under continuous and pulsed light conditions show that in all cases, the electrode is covered with an oriented film, as indicated by the direction of the different phases of the photocurrents. In addition, comparison between native and uncoupled purple membrane enable to make the difference between those photocurrent phases which arise from intraproteic charge displacements and those arising from local pH change.

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5-16

SHAPE INSTABILITY OF A CELL DRIVEN BY LOCAL SOFTENING OF ACTIN NETWORK. A.A. Boulbitch, E. Sackmann

Mechanical behaviour of animal cells is determined by their complicated architecture including bilayer membrane with actin sublayer connected to the cell cytoskeleton. The submembranous actin network has an inhomogeneous structure. Fluctuations in the process of actin polymerisation, local rupture or local dissolution of the actin network give rise in network holes arising. Several models describe the mechanical behaviour of cells and vesicles. Under the strong cell deformation only the lateral tension making the main contribution should be taken into account, while global shape transformation in vesicles containing no actin network is described by curvature elasticity model. In order to describe local deflections of a cell surface the previous approaches are expanded. The equation of the local deflection ψ of the cell membrane is obtained to have the form

$$k\Delta^2\psi + B\Delta\psi + D\psi - U(R)\psi + a_3\psi^2 + a_4\psi^3 = 0$$

with the bending rigidity k, and the membrane parameters B, D, a_3 and a_4. U(R) describes the actin sublayer local softening. The local softening of a submembraneous actin network followed by local decrease in lateral elastic modulus of the membrane gives rise to cell shape instability. Depending on the place on the phase diagram the latter manifests itself as either an invagination, or a bud. The phase diagram of bifurcation contains an isolated point of a soft bifurcation. The shape bifurcation is jumpwise everywhere except the isolated point of soft bifurcation. Estimates show that bifurcation takes place in its vicinity, where it is nearly soft and where the amplitude of a deflection strongly fluctuates with slow kinetics.

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5-17

IMPROVED EXPRESSION OF THE SARCOPLASMIC RETICULUM CA^{2+}-ATPASE IN YEAST. L.Bouneau, F.Corre, T.Menguy, P.Falson, and M. le Maire.

The sarcoplasmic reticulum Ca^{2+}-ATPase is a membrane protein responsible for the relaxation of muscles by decreasing the Ca^{2+} concentration of the sarcoplasm. We have formely described a system for heterologous expression of the rabbit Ca^{2+}-ATPase SERCA 1A in the yeast *Saccharomyces cerevisiae* (Centeno, F., et al., FEBS Lett.354, 117-122 (1994)). The protein is present in a native conformation; it is found in several types of membranes, including the plasma membrane of the yeast. Using a procedure based on a different vector and a different strain of *S. cerevisiae* (Pompon, D., Louerat, B., Bronine, A. and Urban, P. Methods in Enzymologie, 272, 51-64 (1996)), we have now multiplied the yield of Ca^{2+}-ATPase by a factor of 7, so that the total amount of Ca^{2+}-ATPase produced by one liter of culture is about 7 mg. In a crude extract, the Ca^{2+}-ATPase represents about 0.4 % of the total proteins. A microsomal fraction, prepared by differential centrifugation, has a higher content of Ca^{2+}-ATPase (about 1 %), and a Ca^{2+}-ATPase activity indicating that the expressed protein is fully active. Several mutants in putative Ca^{2+} binding sites have been prepared with a similar yield, as well as Ca^{2+}-ATPase with an added poly-his tail which will hopefully be useful for ATPase purification after detergent solubilisation. The present yield of Ca^{2+}-ATPase will allow us to perform a number of biochemical and biophysical experiments which were not feasible with the former expression procedure. In particular, we hope to directly test for Ca^{2+} binding to the native Ca^{2+}-ATPase and the various mutants, using $^{45}Ca^{2+}$.

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5-18

NEURON-CHIP WITH 2000 COUPLING SITES.

M.Brenner, R.Weis, A.vom Felde, E. Bertagnolli, P. Fromherz.

We envisage to apply the method of neuron-silicon junctions [1] to large neuronal nets. We report on an integrated chip made by CMOS technology with a rectangular array of 2048 open field-effect transistors. The size of the gates is about 2 μm. The spacing of the transistors is about 50 μm. An arbitray combination of elements can be read out. The chip is stable in electrolyte. Its function is tested by the attachment of individual leech neurons using poly-lysine. The quality of coupling is similar to that observed with simple chips used in previous studies.

[1] P.Fromherz, Ber.Bunsenber.Phys.Chem. 100 (1996) 1093.

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5-19

INTERACTION OF MYELIN BASIC PROTEIN WITH PHOSPHOLIPID MONOLAYERS. *H. Haas, *A. Gliozzi, R.T. Sorbi, °P. Riccio and P. Cavatorta.

Myelin Basic Protein (MBP) is a 18.5 KDa protein that is believed to play a major role in compacting and stabilizing the multilamellar myelin membrane. In the last years, it has been shown that some metal ions, expecially zinc, inhibit the release of MBP from the membrane during "in vitro" induced demyelinating process. In order to better clarify the molecular aspects of such a behaviour, the interaction of MBP with phospholipid monolayers at the air-water interface and with Langmuir-Blodgett multilayers was studied in a variety of ionic and thermodynamic conditions. For negatively charged phospholipids (phosphatidic acid) a distinct influence of the protein on the phase behaviour, indicated by a well defined shift in the phase transition (π_C) of the lipid, is observed. On the contrary, the isotherms of noncharged monolayers (phosphatidylcholine) are not affected at all. The interaction can be explained with the electrostatic binding of the positively charged protein moiety with the negatively charged lipid headgroups. Protein-acidic lipid multilayers were transferred onto solid substrate and characterized by X-ray reflectivity and circular dichroism experiments. X-ray measurements reveal several minima in the reflectivity curve, indicating a well defined layer structure. The circular dichroism spectra show a marked increase of negative ellipticity in the wavelength region around 220 nm with respect to the protein in water and the growing of a positive band at 190 nm. These results clearly indicate that MBP assume a certain degree of α-elix structure upon binding with lipids.

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5-20

COMPARATIVE STUDY OF ANNEXIN V AND ^{187}TRP MUTANT USING FRAP EXPERIMENTS AND NUMERICAL SIMULATIONS.

L.Cézanne[1], A.Lopez[1], P.Demange[1], J.Benz[2], R.Huber[2] and J.F.Tocanne[1].

Annexin V belongs to a family of extrinsic proteins which bind acidic phospholipids via calcium interactions. It has been shown that Annexin V could crystallize in two possible conformations, ^{187}Trp out or in the third domain, which could inserts in the heads groups region. A mutant of recombinant Annexin V which lacks the ^{187}Trp has been isolated. The influence of wild and mutant Annexin V on the lateral diffusion of NBD-PC and NBD-PS inserted in egg-PC/ brain-PS (9/1) mixtures has been investigated using FRAP technique. The wild and, to a lesser extent, the mutant protein reduce the lateral diffusion of NBD-PC and NBD-PS.

On the ground of numerical simulations of the diffusion of phospholipids in an hexagonal lattice in the presence of obstacles, these observations can be accounted for by a strong and long-lived binding of PS to annexin V and the progressive formation of a 2-D protein network at the water-lipid interface. The insertion of ^{187}Trp in the lipid bilayer seems to be very important for the modification of lateral motion of phospholipids.

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5-21

SIMULTANEOUS USE OF THREE FLUORESCENT PROBES (THE ATPase Trp RESIDUES, COVALENTLY-BOUND FITC, AND THE Ca^{2+} CHELATOR quin2) FOR SPECTROSCOPIC DIFFERENTIATION OF THE SUBSTEPS INVOLVED IN Ca^{2+} DISSOCIATION FROM SARCOPLASMIC RETICULUM MEMBRANOUS Ca^{2+} ATPase. Ph. Champeil.

In stopped-flow experiments, we used quin2, a Ca^{2+}-sensitive fluorescent probe and a high affinity chelator, to trigger the dissociation of the two Ca^{2+} ions initially bound to sarcoplasmic reticulum Ca^{2+}-ATPase which had been previously labelled on Lys515 with fluorescein isothiocyanate (FITC). With appropriate excitation and emission wavelengths, it was possible to monitor the fluorescence changes for these two fluorophores as well as those for the ATPase Trp residues. (i) The changes in quin2 fluorescence allowed us to measure the kinetics of Ca^{2+} dissociation. This dissociation, known to be sequential, was nevertheless found to be apparently monoexponential, implying a ratio of 2 between the rate constants for dissociation of the first and the second Ca^{2+} ion. (ii) The tryptophan fluorescence changes accompanying Ca^{2+} dissociation were faster than the overall quin2 fluorescence changes, at least in the presence of KCl or NaCl. Trp residues thus appeared to mainly monitor the dissociation of the first of the two Ca^{2+} ions to be released. In fact, the detailed kinetics of the Trp fluorescence changes depended on the excitation wavelength, emphasizing the heterogeneity of the Trp residues population. (iii) The accompanying changes in FITC fluorescence were preceded by a significant lag period. These delayed events, revealed by a probe located in the ATPase cytosolic domain, far from the putative location of the Ca^{2+} binding sites, imply that conformational rearrangement of the ATPase occurs after dissociation of the two Ca^{2+} ions.

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5-22

INVESTIGATION OF NERVE MEMBRANE FLUIDITY USING RAMAN SPECTROSCOPY

Maximov G. V., Chatterjee S., Churin A. A., Rubin A.B

Abstract

Raman and EPR spectroscopy of myelinated sciatic nerve and leech neuron was used for investigation of the function of membrane fluidity in the regulation of cell excitability. The correlation between rhythmic excitation and fluidity of axolemma and of neuron cytosome lipids was found. The fluidity changes of axolemma and subcellular lipids depended on the level of extra and intra cellular pCa and pH. It was proposed that during rhythmic excitation, the regulation of the state of plasma and of subcellular organelle membrane of nerve cell could be based on membrane Ca^{2+} / H^{+} changes.

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5-23

INTERACTIONS AND INHIBITION PROCESS STUDIES OF ANTI-VIRAL SURFACTANTS ON VIRAL ENVELOPES.

M. Courregelongue, L. Tamisier and P. Peretti

Enveloped viruses infect cells mainly by receptor-mediated endocytosis. The fusion of the viral envelope within endosomes is acid-pH-dependant and requires high cholesterol/phospholip ratio. Viral envelopes are significantly more ordered than their host cell membranes and cholesterol appears to be a specific requirement for viral stability and infectivity. Surfactants such as cyclopentenone prostaglandins and amphiphilic polymers are known for their antiviral effects but the mechanisms of inhibition have not been yet identified. To characterise the interactions between these surfactants and viral membranes, we used in situ imaging and fluorescence microscopy combined with Langmuir film balance techniques. When a mixture of DPPC/cholesterol is spread at the air-water interface, a direct gas/condensed-liquid phase transition is observed. The missing of the expanded-liquid phase is due to cholesterol-induced condensation effects. Incorporation of prostaglandin in the mixed monolayer exhibited a new condensed/expanded-liquid phase transition in the surface pressure isotherms. Using a NBD-PC fluorescent probe, we observed that the surfactant induces the formation of organized domains in expanded-liquid phase. These large domains result probably from the interactions of prostaglandins with phospholipid polar heads. This fluidization process may explain the anti-viral properties of these surfactants since fusion was shown very dependant on the condensation state of the viral envelopes Our present studies are defining the interactions responsible for the modifications of the envelope organisation.

On the hypothesis of selective interactions between some polymeric surfactants and condensed membranes, this mechanism of inhibition may provide a new general therapeutic approach on enveloped viruses such as HIV and many oncogene viruses.

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5-24

GIANT UNILAMELLAR VESICLES : MODEL FOR BIOLOGICAL MEMBRANES ?

S. Cribier, I. Sagot and Ph. F. Devaux.

We have used Giant Unilamellar Vesicles (GUVs, 10 to 150 microns in diameter) as model systems for biological membranes. GUVs are produced by an AC electric field controlled swelling of a lipid film.

Significant shapes changes were obtained by osmotic pressure modifications, pH gradients, or by addition (depletion) of phospholipids and were monitored by optical microscopy. They mainly consist of the shape transformation of one vesicle into dumbbell-shaped vesicles. The effect of different lipid compositions on these deformations have been studied, in particular the effects of charged phospholipids or cholesterol.

In addition, the use of fluorescent phospholipid analogs allowed us to better visualize the membrane, as well as to test the connectivity of the vesicles after deformation using the FRAP experiment (Fluorescence Recovery After Photobleaching). Under certain conditions, adhesion of liposomes was observed, without vesicle fusion being observed.

These GUVs have been used in experiments investigating the possible role of tetrameric Annexine II during fusion. GUVs and LUVs (Large Unilamellar Vesicles, 100nm in diameter) containing both PS (15%) are able to fuse when Annexine II is added in presence of calcium and phophorylated *in situ*.

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5-25

INTERACTION WITH MEMBRANE AND INTERNALIZATION BY MACROPHAGES OF A T-CELL EPITOPE LINKED TO A FUSION PEPTIDE. A. Delmas, R. Maget-Dana, C. Favard, D. Lelièvre, P. Vigny, and Y. Trudelle.

The modification of T-cell epitopes by a hydrophobic sequence representing residues 113 to 131 (F) of the fusion protein of measles virus enables the induction of cytotoxic T-cell responses. It has been proposed that other hydrophobic modifications, such as derivatisation by fatty acid, facilitate the *in vivo* induction of cytotoxic T-cell responses *via* the interaction of the lipopeptide with plasma membrane.
In this report, we describe the interaction with membranes of a chimeric peptide composed of the fusion peptide F linked to the T-cell epitope 81-95 from the M2 protein of respiratory syncytial virus. *In vitro*, the penetration of the chimeric peptide was studied in a lipid monolayer as a model of membrane. In addition, the conformation of the chimeric peptide in the presence of phospholipids will be described. *In situ*, its interaction with living macrophage-like cells was followed by microspectrofluorometry. The chimeric peptide interacted with the plasma membrane and was internalized after 2 hours incubation. The internalization was inhibited at 4°C and at 37°C in the presence of cytochalasin B.
The results presented here support the hypothesis that hydrophobic modification of a T-cell epitope by a fusion peptide enables it to interact with membranes and consequently leads to its internalization by macrophages for *in vivo* CTL induction of T-cell responses.

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5-26

COMPARATIVE ELECTRIC POTENTIAL STUDIES ON MULTI-LAYERED MODEL MEMBRANES COMPOSED OF CHARGED LIPIDS AND POLYELECTROLYTES A. Diederich, G. Bähr and M. Winterhalter

The vibrating plate method (VP) and inner field compensation technique (IFC) were combined to study the adsorption of pentalysine, polylysine and DNA on Langmuir films and Black Lipid Membranes (BLM) composed of charged and zwitterionic lipids. The monolayer experiments were carried out at a lateral pressure of 30 mN/m and constant area. It was found that in case of binary mixtures of Phosphatidylcholine and Phosphatidylserine the adsorption of pentalysine causes similar changes in the electric potentials for both model-membranes. Apparent binding constants were determined for various lipid mixtures.

The layer by layer adsorption of polylysine and DNA to Phospho-serine membranes causes the transmembrane voltage of both model membranes to change in a fashion which strongly suggests that the growing of multilayered structures can be monitored in situ.

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5-27

BACTERIORHODOPSIN CARRIES OUT A MOLECULAR SORTING IN DLPC/DSPC PROTEOLIPOSOMES. F. Dumas, M.C. Lebrun, M. Sperotto, O.G. Mouritsen and J-F Tocanne

It has recently been shown that a transbilayer protein like bacteriorhodopsin (BR) was able to modify the phase properties of phosphatidylcholine bilayers (Perochon et al.,1993). These modifications were attributed to modifications of the length of the acyl chains of the lipids witch are in contact with the protein in order to achieve the best matching between the hydrophobic length of the protein and the hydrophobic thickness of the lipid bilayer.
The aim of this research was to investigate the possibility that a transbilayer protein, via this hydrophobic matching principle can perform molecular sorting of the lipids.
Still using bactriorhodopsin, fluorescence energy transfert experiments were carried out using the NBD chromophore attached to lipid polar head as donor and retinal in BR as acceptor. BR was reconstituted in DLPC/DSPC mixture where the phase diagram is well established. Combined results from computer simulations and fluorescence experiments provide evidence that at low and moderate temperatures in the gel-gel and the fluid-gel coexistence region, BR is solvated by the short chain lipid DLPC. At high temperature, in the fluid-fluid region, at least the first lipid layer around the protein would be enriched in the long chain lipid DSPC detrimental to DLPC.

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5-28

ELECTRO-OPTICAL ANALYSIS OF BACTERIAL RESPONSES TO ELECTRIC PULSE. N. Eynard, and J. Teissié.

Analysis of the turbidity signals showed that electropermeabilization of cell enveloppe could be observed by electrooptics studies with a fast time resolution.
During the application of an electric field, the rod-like shaped *E. coli* was oriented along the direction of electric field (EF). When field parameters were adjusted, EF induced enveloppe permeabilization with associated electrotransformation. EF could caused a loss in cell viability.
Kinetic studies of *E. coli* orientation and permeabilization were made by the analysis of the change in transmitted light during the pulse. Comparison of kinetic analysis with quantitative studies led us to obtain the following conclusions for bacteria:
- orientation controlled all other responses of the cell to vectorial effects of EF (i.e. permeabilization). During the orientation phase, no effect could be observed because the part of the cell submitted to the field changed. Only when cells were oriented, membrane permeabilization was effective.
- Enveloppe permeabilization obeided different processes for small molecules (ions or metabolites) and macromolecules (plasmids). At low permeabilising EF, permeabilization extent was very slow, and only small molecules could be exchanged between internal and external medium (no transformation occured). At higher EF, permeabilization and orientation were in the ms range and efficient cell permeabilization, and associated transformation, occured.

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5-29

THE TWO LOCATIONS OF CHOLESTEROL SULFATE IN DIMYRISTOYLPHOSPHATIDYLCHOLINE MEMBRANES REVEALED BY NEUTRON DIFFRACTION AND MOLECULAR MECHANICS SIMULATION. C. Faure, M. Laguerre, W. Néri and E.J. Dufourc.

Cholesterol sulfate (CS) and cholesterol (CH) are chemically alike and yet, a variance in the delicate balance of CS versus CH in spermatozoid membranes or stratum corneum may alter fusion of the former or trigger a disease of the latter. It has recently been proposed that the higher hydrophilicity of CS could be partly responsible for these troubles. This property would result in a different vertical location of the steroid in the bilayer (Faure et al., 1996). Such an hypothesis was assessed by neutron diffraction using ring-deuterated and unlabelled CS embedded in oriented dimyristoylphosphatidylcholine (DMPC) membranes. Proton-deuterium contrast methods afforded the location of the labels and showed that, for the three studied temperatures i.e., 10, 23 et 50°C, CS can occupy two distinct sites in these bilayers. About half the CS is embedded as CH, that is with the polar moiety at the level of the fatty acyl carbonyl groups of DMPC. The other half is 6 Å upper in the bilayer, in the aqueous compartment, such that the sulfate group is in favorable electrostatic interaction with the quaternary ammonium of choline lipid headgroup. These experimental results were reinforced by an energetic minimization realized on DMPC lamellae containing the same CS content as for neutron experiments (33 mol%). The steroid was initially positioned as CH, i.e., embedded in the bilayer. In the optimal energetic situation, CS molecules are found to be evenly distributed between the two locations as reported by neutron diffraction.

Faure C, Tranchant JF , Dufourc EJ (1996), Biophys. J. 70: 1380-1390

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5-30

DEPENDENCE OF Na-Ca-EXCHANGE CURRENT AMPLITUDE OF SECRETORY CELLS MEMBRANE ON EXTRACELLULAR Ca^{2+} CONCENTRATION. N. Fedirko, M. Klevets, V. Manko.

We have identified the Na-Ca-exchange current (I_{NaCa}) which was inward at physiological Na^+ gradient and outward - at opposite. Continueing of this study we investigated the dependence of exchange functioning on $[Ca^{2+}]_e$ by mean of registration the I_{NaCa} at indicated above Na^+ gradients. I_{NaCa} of isolated cells of the salyvary gland in Chironomus larvae was registered by mean of voltage clamp method in the conditions of intracellular perfusion in response to membrane hyperpolarization from -20 to -60 mV.

It was founded out that amplitude of outward I_{NaCa} was increased by 16.1 % on the decrease in $[Ca^{2+}]_e$ from physiological (1.76 mM) to 1 mM. When $[Ca^{2+}]_e$ was diminish to 0.1 mM the amplitude of outward I_{NaCa} decreased by 23.8 % compare with the control. However, outward current was registered even in the conditions of nominally calcium-free solution (without helators). It did not dissapear during the addition of 10 mM EDTA to nominally calcium-free solution. Dependence of inward I_{NaCa} on $[Ca^{2+}]_e$ showed different character: increasing of $[Ca^{2+}]_e$ from 1 to 5 mM evoked slow rise of it amplitude. Increasing of $[Ca^{2+}]_e$ to 10 mM did not reflected on the current amplitude.

We came to the conclusion that increasing of I_{NaCa} as a result of diminish in $[Ca^{2+}]_e$ to 1 mM can be explained as a recovery of exchanger from the state that remaind of substrate inhibition of enzyme activity, since at the conditions of outward I_{NaCa} registrations the exchanger bind Ca^{2+} at outside of membrane. Hyperbolic dependence of I_{NaCa} amplitude on $[Ca^{2+}]_e$ testify on saturation of exchanger cation binding site by cytosolic Ca^{2+} because in the conditions of registration of inward I_{NaCa} the exchanger bind Ca^{2+} at inside of membrane.

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5-31

CHARGE TRANSLOCATION BY THE K^+ TRANSPORTING KDP-ATPASE FROM E. COLI. K. Fendler, S. Droese, K. Altendorf and E. Bamberg.

Charge translocation by the Kdp-ATPase of *Escherichia coli* was investigated by adsorption of proteoliposomes to a planar lipid membrane. The proteoliposomes were prepared by reconstitution of purified Kdp-ATPase into liposomes prepared from *E. coli* lipids. The protein was activated by a ATP concentration jump produced by photolysis of a protected derivative of ATP, caged ATP. Charge translocation was measured with a time resolution of 15-40ms. Stationary currents demonstrated the continuous pumping activity of the enzyme. Control measurements with the potential-sensitive dye $DiSC_3(5)$ showed a negative potential inside the proteoliposomes after activation with ATP.

The measured electrical signals as well as the dye measurements correspond to the transport of positive charge to the intracellular face of the protein. The electrical signal was increased when K^+ was inside the proteoliposomes ($K_{0.5} \approx 50$ μM) and was inhibited by vanadate. These experiments demonstrate the electrogeneity of the Kdp-ATPase in a purified reconstituted system.

Transient electrical signals were measured also in the absence of K^+ for the wild type protein as well as for a low K^+ affinity Kdp-ATPase mutant (Q116R). Therefore, we have to conclude that the Kdp-ATPase can perform an electrogenic reaction even in the absence of K^+. We propose that this reaction corresponds to the translocation of unoccupied negative binding sites from the cytoplasmic to the extracellular side of the protein.

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5-32

METASTABILITY OF A CIRCULAR O-RING INDUCED BY INTRINSIC CURVATURE T. Charitat and B. Fourcade

An o-ring takes spontaneously the form of a chair when subjected to strong enough torsion. This state is metastable since work has to be done to return to the circular shape. We show that this metastable state exists for a Hamiltonian where curvature and torsion are coupled by an intrinsic curvature. If the o-ring is constrained to be planar (2d case) this metastable state exists if the ratio α=C /A of the torsion elastic constant to the bending elastic constant is less than 0.66. In the 3d case, where the o-ring is not planar, our variational approach shows that the critical value of α is 0.9. In both cases we show that this metastable state is characterized by a kink-antikink pair separated by two circular segments. We discuss this analysis in the context of DNA minicircles where histones like proteins like MC1 are known to bind preferentially to bend segments and to induce conformationnal changes.

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5-33

NONLINEAR STABILITY OF BIOMEMBRANES WITH SURFACE CHEMICAL REACTIONS.

D.Gallez, A.De Wit and M.Kaufman
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The influence of a chemical reaction on the dynamical behaviour of a biomembrane is of significant importance in many biological applications. The dynamics of the interaction of a biomembrane on a solid support is followed until membrane rupture or formation of local contacts (periodic patterns). A surface chemical reaction between insoluble surfactants molecules (receptors) on the free surface of the membrane and binding sites (ligands) on the solid substrate is now considered Asymptotic expansion of the equations for fluid motion with van der Waals, capillary and Marangoni forces allows to obtain a model with 3 non-linear evolution equations describing the dynamics of the surface deformation, and the kinetics of free and bound receptors. Chemical and hydrodynamic instabilities are predicted and simulated numerically with different stability regimes. For a simple surface reaction, the concentration of receptors follows the deformation of the surface; for a non-linear surface reaction (affinity enhanced at small distances), a clustering of receptors is observed at the local points of contacts. When the Marangoni coupling is increased, a completely new regime is obtained, where the receptor concentration oscillates, delaying the rupture (or contact) time by several order of magnitude. A critical density of binding sites is also necessary to observe this time delay.Possible applications to agglutination of erythrocytes by polymers or to receptor clustering in immunology will be discussed.

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D.Gallez and W.T.Coakley, *Far-from-equilibrium phenomena in bioadhesion processes*, Heterogeneous Chemistry Reviews, **3**, 443-475 (1996).

5-34

EFFECT OF DODECYL MALTOSIDE DETERGENT ON G_t ACTIVATION BY RHODOPSIN STUDIED BY FLUORESCENCE SPECTROSCOPY.

J. Marrón[1], D. García-Quintana[1], J. Manyosa[1] and P. Garriga[1,2]

Rhodopsin is the G protein-coupled receptor responsible for scotopic vision. Upon light absorption by its chromophoric group (11-*cis*-retinal), rhodopsin undergoes a conformational change to the active Meta II intermediate, which binds and activates the G_t protein, on the cytoplasmic surface of rod retinal disks. These processes are clearly dependent on the lipidic environment of rhodopsin in rod membranes and the status of the lipid bilayer.

We have studied the effect of adding dodecyl maltoside (DM) detergent to native rhodopsin in membranes, on G_t protein activation, using fluorescence spectroscopy. In the assay, the increase of the intrinsic $G_{\alpha t}$ subunit fluorescence, as a result of the activation process, is monitored at 340 nm after sample irradiation at 295 nm. Two parameters have been analysed: number of G_t activated molecules (measured as the maximum fluorescence gain) and initial activation rate (slope determined from the linear regression of the first 60 s data points). Both parameters show an analogous trend with increasing concentrations of detergent in the sample: (i) a first increase at low detergent concentrations, up to about 0.01% DM (close to its CMC value), specially marked on the initial activation rate. This behaviour would be reflecting an increase in membrane's fluidity; (ii) a decrease at higher concentrations related to micelle formation, process which would impaire rhodopsin-G_t interaction and possibly denature receptor's exposed domains.

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5-35

MAGNETIC NANOPARTICLES AND PHOSPHOLIPIDS MEMBRANES INTERACTIONS.

F. Gazeau, A. El Abed, C. Menager, M. Boynard

Colloidal solutions of magnetic nanoparticles (ferrofluids) have been developed for biomedical applications as - contrast agents for NMR imagery - magnetic separation of cells or - for the induction of a local hyperthermia under high frequency field which enables a magneto- thermocytolysis. These applications require a cellular targeting and thus a precise knowledge of the interactions between the magnetic nanoparticles and the cellular membrane. A model system can be built from the association of ferrofluids with lipid bilayers vesicles, either by encapsulating magnetic particles inside the vesicle or by trapping the particles specifically inside the membrane or outside the bilayer. These biocompatible magnetic vesicles could be used as vectors of drugs. It is shown for giant ferrovesicles that an external magnetic field flattens the thermal undulations and induces a shape deformation which can be analyzed to determine elastic constants of membranes. We characterize the interactions between ionic magnetic nanoparticles and phospholipid layers :

- by studying phospholipid monolayers at the air - aqueous ferrofluid interface using fluorescence microscopy combined with Langmuir film balance techniques. The surface pressure vs. molecular area isotherm of different phospholipid monolayers is modified by the adsorption of magnetic nanoparticles only in the condensed phase.
- by characterizing the deformability of magnetic vesicles using ultrasound backscattering.

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5-36

MORPHOLOGY AND BENDING ELASTICITY VARIATIONS OF GIANT LIPOSOMES INTERACTING WITH AMPHIPATHIC PEPTIDES.

C. Gerbeaud, P. Méléard, J. Dufourcq.

Natural peptides can be used as defense against aggressions from outside, selective antibacterial properties being recognized sometimes. These molecules are generally amphipathic, explaining their strong affinity with natural or model membranes. To understand how they modify the membrane behavior, we studied melittin and gramicidin, two natural peptides, and L_iK_j, a synthetic peptide characterized by a well-defined hydrophobic/hydrophilic balance and a variable length. These peptides are known to strongly interact with bilayers inducing a large disturbance in the lipid molecular arrangement. Giant unilamellar liposomes from stearoyl oleoyl phosphatidylcholine (SOPC) obtained by electroformation are used as model systems to study the macroscopic consequences of the peptide addition in the membrane.

These perturbations can be observed when the peptide concentration is homogeneous inside the membrane and constant in time. Using this approach we measured the bending elasticity k_C of SOPC bilayers as a function of the lipid/peptide membrane ratio (R_i). We show that the perturbation induced by the peptide depends both on R_i and on the molecular structure of the peptide.

A more significant experiment with regard to the mode of action of those peptides was to use giant SOPC liposomes which after formation can be transferred using micromanipulation into a solution of a water soluble peptide (melittin or a short L_iK_j). The peptide concentration within the bilayer is now time dependent and should be related to a bilayer asymmetry. Macroscopic changes appear rapidly, showing an increase of the vesicle surface/volume ratio resembling to budding.

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5-37

pH TRIGGERED ANCHORING OF SOLUBLE PROTEINS TO MEMBRANES USING THE DIPHTHERIA TOXIN TRANSLOCATION DOMAIN. D. Liger, P. Nizard, C. Gaillard and D. Gillet.

We have studied the possibility to anchor human interleukin 2 (hIL2) and murine interleukin 3 (mIL3) to the surface of liposomes using the diphtheria toxin translocation domain (T domain). The T domain may exist in two distinct well-characterized states. At neutral pH it is soluble while at acidic pH it inserts in biological membranes, in an irreversible manner.

We have constructed two fusion proteins T-hIL2 and T-mIL3 in which hIL2 or mIL3 are fused to the C-terminus of the T domain. Proteins were produced in *E. coli.*

Recognition of the hIL2 receptor or the mIL3 receptor by the corresponding recombinant proteins was demonstrated in binding inhibition experiments.

pH-triggered insertion of the recombinant proteins in membranes was first monitored by measurements of the permeabilisation of liposomes (PC/PA 9/1) to protons, as the inserted T domain forms a channel selective for small monovalent cations. Insertion was inhibited by anti-diphtheria toxin antibodies but not by anti-hIL2 or anti-mIL3 antibodies.

Finally, anchoring of both interleukins to the membrane of liposomes was assessed by specific antibody recognition in a liposome enzyme-linked immunofiltration assay (lipo-ELIFA).

Our results show that the T domain fused to the N-terminus of a given protein can function as a pH-sensitive membrane anchor for that protein.

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5-38

MEMBRANE ORGANISATION ALTERATIONS MODULE THE ELECTROTRANSFER OF MACROMOLECULES INTO MAMMALIAN CELLS. M. Golzio, J. Teissié , M.P Rols

Electrotransfer of small molecules (<4kDa) depends on the pulsing conditions (field intensity, pulse duration, number of pulses). They pass through the membrane by free diffusion during and in the minutes after the electric treatment. Electropermeabilization to macromolecules is different. Plasmid DNA must be present during the electric pulse application and ms time range pulse duration is needed to transfect the cell.

The mechanism of plasmid DNA transfer appears to be a multistep one. First, the plasmid would interact with the cell and insert into the membrane during the electric treatment. After pulsations, the plasmid would be translocated into the cytoplasm. As these macromolecules should interact with the membrane, we study the effects of membrane organisation on their transfer.

Osmotic pressure controls the membrane order. The effects of the osmotic pressure during the electric treatment and during the 10 minutes following it were investigated. In hypotonic media, the repulsive forces (i.e. undulation forces) decrease. The energy barrier preventing the DNA interaction with and across the membrane is reduced. A higher transfer takes place.

Ethanol known to decrease membrane order also decreases plasmid DNA transfer. Conversely, lysophosphatidylcholine known to increase membrane order, increases electrotransfection. Interactions between plasmid DNA and membrane phospholipids seem to be a key step for electrotransfection mechanism.

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5-39

CHARGE TRANSLOCATION BY THE Na-Ca EXCHANGER INDUCED BY Ca^{2+} CONCENTRATION JUMPS. M. Kappl, G. A. Nagel and K. Hartung.

Kinetic properties of the Na-Ca exchanger from heart muscle cells were investigated by combining the „giant patch clamp" technique with Ca^{2+} concentration jumps generated by the photolysis of a cleavable Ca^{2+} chelator (DM-nitrophen). In the Na-Ca exchange mode i.e. with 100 mM Na^+ on the extracellular side a saturating Ca^{2+} concentration jump (ΔCa^{2+} = 100µM) elicits a transient inward current which decays to a plateau with τ = 0.7 ms (21 °C). The rise-time, distorted by the laser flash, is less than 0.1 ms. In the absence of extracellular Na^+ with 5 mM extracellular Ca^{2+} , i.e. in the Ca-Ca exchange mode, a Ca^{2+} concentration jump elicits a transient but no stationary current. At positive membrane potentials the amplitude of the peak and stationary current is reduced and the decay time constant increased. The opposite is observed at negative voltages. These results indicate that 1) Ca^{2+} and Na^+ translocation are electrogenic, 2) outward translocation of Ca^{2+} is associated with outward movement of negative charge and inward translocation of Na^+ is associated with the inward movement of positive charge 3) Ca^{2+} translocation is not rate limiting. In further experiments the magnitude of the Ca^{2+} concentration jump has been changed. The rise time is very little changed by reducing the Ca^{2+} concentration. In the Ca-Ca exchange mode the peak amplitude is reduced and the decay time constant increased but the amount of charge is fairly independent of the magnitude of the Ca^{2+} concentration jump. In the Na-Ca exchange mode a comparable increment of the decay time constant is observed but in contrast to the Ca-Ca exchange mode the Ca^{2+} dependence of the peak current cannot be described by Michaelis-Menten kinetics.

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5-40

WATER MOLECULES IN THE PROTON PATHWAY OF THE LIGHT-DRIVEN PROTON PUMP BACTERIORHODOPSIN T. Hauß, G. Papadopoulos, S. A. W. Verclas, G. Büldt and N. A. Dencher

Bacteriorhodopsin (BR) is an integral membrane protein that functions as a light-driven proton pump, establishing a proton gradient across the membrane. This proton gradient is then used to energize metabolic processes. By time resolved optical spectroscopy we have investigated the proton pump activity of BR at reduced hydration, i.e. the kinetics of the photocycle and the proton pump stoichiometry. These spectroscopic results and the three dimensional model of BR suggest that water is an essential part of the proton pathway. Previous results from neutron diffraction revealed at low humidity, at whith no proton transport across the membrane can be observed, that about 3-4 water molecules are tightly bound in the proposed proton pathway, whereas 10-20 water molecules can be placed in the proton pathway in the atomic model of BR with different model calculations. Here we present results from extended neutron diffraction experiments with BR samples at more physiological hydratation conditions, i.e. the samples were held at 57%, 86% and 94% rel. humidity. The measurements were performed on the V1 membrane diffractometer at the Hahn-Meitner-Institut in a $\theta - 2\theta$ geometry. The number and locations of water molecules in projection onto the membrane plane were determined from differences in neutron diffraction intensities of samples kept in H_2O and D_2O atmospheres by a novel model calculation aproach. This model calculation circumvents limitations of the difference Fourier method and leads to more reliable results and will be compared with results from the difference Fourier synthesis.

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5-41

STRUCTURAL TRANSITIONS OF LIPID MEMBRANES CLOSE TO THE CHAIN MELTING TRANSITION

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The change of the inner energy of lipids with temperature is related to lipid volume and area changes, respectively. Close to the chain melting transition of lipid bilayers volume and enthalpy fluctuations are to a good approximation proportional. This allows to relate the membrane excess heat capacity with the lipid volume and area compressibility and to a first order to membrane bending moduli, if the area fluctuations of the two monolayer are assumed to be mainly uncoupled. Thus, compressibility and elasticity display pronounced maxima at the chain melting transition. This has important implications in respect to the induction of structural transitions in membranes that are linked to differential changes of the membrane monolayer areas, namely to curvature or ripples. Chain melting and structural changes may couple close to the main transition. Vice versa, structural changes effect the excess heat capacity profiles, resulting in curves that display more than one heat capacity maximum in a single lipid membrane, although all peaks may be related to the chain melting.

This concept has been used to simulate our recent findings of structural transitions close to the main transition in charged lipid membranes, involving curvature changes. These changes are driven by osmotic and/or electrostatic effects that favor a complex three-dimensionally extended membrane network over lipid vesicles in the chain melting regime. The resulting melting profiles display three ΔC_p-maxima. One may tentatively also relate this effect to more complex transition phenomena close to the melting transition as are the lipid pretransition or the sub-main transition.

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THE INTERACTIONS WITH LIPIDS GOVERN THE EFFICIENCY OF VECTOR PEPTIDES. N. Van Mau, L. Chaloin, P. Vidal, G. Divita and F. Heitz

The interactions with lipids of two series of primary amphipathic peptides were studied by the monolayer method. These peptides are built of the association of a hydrophilic sequence (NLS) with a hydrophobic one which is either a signal peptide (Series 1) or a fusion sequence (series 2) separated (1a and 2a) or not (1b and 2b) by a linking peptidic motif. Peptides 1a, 1b and 2a were shown to be internalized into cells with a final nuclear localization while 2b remains membrane associated. A monolayer study reveals that peptides 1 penetrate into the lipid hydrophobic cores and interact with lipids through hydrophobic interactions whatever the nature of the polar headgroups (PG, PS or PC). On the contrary, peptides of the series 2 do not penetrate into PC membranes. This was assessed from fluorescence measurements (there is a Trp residue within the hydrophobic sequence) and a monolayer study confirm this point. Within this series the linking sequence governs the lipid-peptide interactions. Its absence in series 2 favours the lipid-peptide interactions compared to the peptide-peptide ones while in all other cases, when monolayer penetration occurs the hydrophobic interactions arising from peptide - peptide or peptide - lipid interactions remain almost identical. These observations can be related to the cellular internalization efficiency and may constitute one of the basis for the understanding of the mechanism of cellular uptake and thus for the future design of drug carriers.

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MODÈLE CYBERNÉTIQUE DE LA MEMBRANE PLASMATIQUE DU BÂTONNET. S. Herman, C. Dimoftache

En partant d'un modèle électrique de la membrane plasmatique et de la description mathématique des processus impliqués dans l'établissement du potentiel membranaire (les courants ioniques par les canaux et les pompes), on a conçu un modèle cybernétique. Nous avons suivi les conditions de régime stationnaire et la succession des événements déclenchés par la fermeture des canaux ioniques contrôlés par le GMP cyclique, dépendant de l'intensité du stimulus. Nous avons déduit les expressions mathématiques des courants par les canaux ioniques et par la pompe d'échange $Na^+/Ca^{++}/K^+$, de l'article externe (AEB); nous avons de même inclus dans le modèle la pompe de Na^+/K^+ et le canal de K^+ de l'article interne du bâtonnet.

Les grandeurs d'entré du système cybernétique sont: 1. le nombre des canaux ioniques ouverts, donc la probabilité d'ouvrir (p) multipliée par le nombre total des canaux (C_0) et 2. la concentration cytoplasmique des ions de Ca^{++}. Les grandeurs de sortie sont le potentiel membranaire (V) et la contribution à la modification de la concentration cytoplasmique des ions de calcium (ΔCa^{++}).

Il en résulte de notre modèle que la variation des tous les courants ioniques induit une réaction positive qui mène à l'accélération de la réponse électrique de la membrane, et, en même temps, une réaction négative qui limite l'hyperpolarisation. L'hyperpolarisation elle même cause l'évolution des courants en cette direction. Les mécanismes membranaires interviennent dans la diminution de la sensibilité de la cellule photoréceptrice à des stimulus de grande intensité et, par conséquent, dans l'adaptation à la lumière, par l'amoindrissement de la concentration cytoplasmique des ions de calcium, qui contrôlent, en bien mesure, les processus biochimiques de l'AEB.

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5-44

INCORPORATION OF BACTERIORHODOPSIN INTO SOLID SUPPORTED MEMBRANES. C.Steinem, A.Janshoff, A.Michalke, A.Eing, F.Höhn, M.Sieber and H.-J.Galla

Introduction: Solid supported membranes are discussed as a useful tool in modelling biological systems and now serve as a basis for biosensor devices as well. We incorporated the protein Bacteriorhodopsin(BR) into such an artificial system and investigated its current response on the exposure to light indicating the protein's activity.

Methods: Phospholipid vesicles, containing 1mol% of BR were fused on a hydrophobic monolayer of DMPTE (1,2-Dimyristoyl-*sn*-glycero-phosphatidylcholine) formed on a gold surface by a self-assembly technique. Impedance spectra of the system provided information about the success of the preparation. Transient currents, indicating the electrical response on light, were measured using a high-gain amplifier.

Results: It was demonstrated, that BR could successfully be reconstituted into the artificial membrane arrangement. The dependence of the time constants of the transient currents on light intensity as well as the influence of the proton carrier FCCP (Carbonylcyanid-p-trifluoromethoxyphenylhydrazone) was proved in our experiments.

Conclusions: The stability of the solid supported bilayers and the pump activity of BR remaining constant for about 24 h make the system suitable for biosensor developments. It is now our aim to show how the current response depends on the variation of the protein's lipid environment and on chemical changes in the molecule itself.

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5-45

PHOSPHOLIPASE A_2 ACTIVITY ON UNSATURATED AND BRANCHED PHOSPHATIDYLCHOLINE LARGE UNILAMELLAR VESICLES. P. Høyrup and T. Hønger

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of phospholipids. The temperature dependence of the enzymatic activity in one-component systems of large unilamellar vesicles (LUV) of palmitoyl-oleyl-phosphatidylcholine (POPC), stearoyl-oleyl-phosphatidylcholine (SOPC), di-arachidonoyl-phosphatidylcholine (DAPC), and diphytanoyl-phosphatidylcholine (DPhPC) was studied using the intrisic PLA_2 fluorescence and static light scattering.

The onset of rapid hydrolysis after a period of slow hydrolysis (the lag time) was identified with the observation of sudden increase in flourescense. In the low-temperature interval of 283 K and 313 K the logarithm of the lag time was found to be linearly dependent on the inverse of the temperature. For all of the substrates studied, the lag time exhibits a minimum at 313 K. Above this temperature, the lag time increases, possibly due to denaturation of PLA_2.

These results suggest, that the hydrolysis during the lag-time, at temperatures where neither the lipid substrate nor the enzyme exhibit thermal induced transitions, is an activated reaction, with an activation energy strongly modulated by the lipid substrate. The activation energies increased in the order of DPhPC < DAPC < POPC < SOPC, and were between 0.4 and 1.6 $\times$ 10^{-19} J.

The temperature dependence of the lag time found in this study supports a previously proposed relationship between enzymatic activity of PLA_2 and bilayer bending rigidity.

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5-46

STRUCTURAL SPECIFICITY OF INTERACTION OF THE ANTIMICROBIAL PEPTIDE ALAMETHICIN WITH BILAYER AND NON-BILAYER FORMING LIPIDS.

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Alamethicin is an antimicrobial antibiotic capable of pore formation in lipid membranes. The activity of the peptide is dependent on the membrane lipid composition. Depending on the lipid/peptide molar ratio alamethicin may adsorb at the hydrophilic interfaces of the lipid bilayers or penetrate into their hydrophobic regions. The mechanism of alamethicin channel formation has been explained by the lateral aggregation of the amphipatic α-helical peptide monomers into bundles of helices spanning the membranes (barrel-stave model). While this mechanism has been accepted for the interaction of alamethicin with bilayer forming membrane components, little is known about the effect of the peptide on the structural phase behavior of non-bilayer forming lipids. In this study, the incorporation of alamethicin in fully hydrated dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) is investigated by means of time-resolved synchrotron X-ray diffraction. The two zwitterionic phospholipids differ in the chemical structure of their hydrophilic head groups, which determines differences in their hydration, hydrogen bonding ability and phase behavior. Structural changes with the liposomes of the non-bilayer forming lipid DOPE are found to be dependent on the concentration of alamethicin in the aqueous phase. At a critical peptide concentration, a transition from inverted hexagonal to cubic phase structures is established. Within the investigated region of peptide concentrations, the phase behavior of the bilayer forming lipid DOPC is not affected by the presence of alamethicin. The obtained results demonstrate the importance of intermolecular interactions for the accommodation of alamethicin by liposomes.

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5-47

VOLTAGE-DEPENDENT VECTORIAL TRANSLOCATION OF POLYSIALIC ACID CHAINS ACROSS MEMBRANES. Tadeusz Janas, Teresa Janas, Frederic A. Troy and Henryk Krajiński.

The membrane-associate CMP-Neu5Ac:poly-a-2,8-sialosyl sialyltransferase (polyST) complex from neuroinvasive *E. Coli* K1 catalyses the synthesis, transmembrane translocation and assembly of polysialic acid (polySia) capsule. To determine the molecular mechanism of translocation of polysialic acid chains across the inner membrane, we have developed an *in vivo* labeling system using spheroplasts prepared from K1 cells that are unable to degrade sialic acid (nanA4 mutation). After pulse-labeling the spheroplasts with ^{14}C-Neu5Ac, synthesis and translocation were followed kinetically. Our results show that both the membrane protomotive force ($\Delta\mu H^+$) and the transmembrane electrical potential gradient ($\Delta\psi$) are actively involved in the transmembrane translocation of polySia chains. A computer simulation of the polySia translocation across membranes was also performed. The derivation of the flux equations was based on the equivalence electrical circuit of the investigated system. The obtained results are shown in the form of three-dimensional graphs. These results indicate that the magnitude of the polysaccharide flux depends on the degree of polymerization and the membrane conductance, and the shape of the graph surfaces is modulated by the water solution conductance, the scan rate of the applied potential and the membrane capacitance. The data continue to emphasize the complexity in polySia synthesis, export ans assembly in prokaryotic cells, and underscore the probable complexity to be found in regulating surface expression of polySia in eukaryotic cells.

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5-48

BISTABILITY OF NEURON-TRANSISTOR.

M. Jenkner, P. Fromherz.

In a neuron-transistor the action potential of a nerve cell affects directly the source-drain current of a insulated-gate field-effect transistor [1]. Crucial for the strength of coupling is the current through the membrane in the attachment site which drops along the seal resistance between membrane and gate. Two types of systems were observed with biphasic (A-type) and monophasic (B-type) response [1, 2]. We report here on a reversible transition between an A-type and a B-type junction. A Retzius neuron of the leech is attached to a transistor by poly-lysin. Action potentials are elicited by current injection through an impaled microelectrode. By lowering and lifting the electrode the response of the junction is switched from a weak biphasic to a strong monophasic response and vice versa, respectively. The two states of the junction can described by a small and a large conductance of the membrane in the adhesion site with about 0.3 mS/cm^2 and 3 mS/cm^2. Thus the mechanical deformation of the cell membrane by the electrode induces a switching process of the membrane conductance in the adhesion site. The nature of the nonlinearity involved is unknown.

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[2] R.Weis, P.Fromherz, Phys.Rev.E 55 (1997) 877.

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5-49

ADHESION BETWEEN GIANT LIPOSOMES: AN OPTICAL MICROSCOPY STUDY. F.M. Menger, J.S. Keiper, and S.J. Lee.

Liposomes are commonly used as model systems in the study of important cell processes such as fusion and adhesion. Investigation has primarily focused on submicroscopic liposomes (diameters ≤ 1 μm) which are examined using spectroscopic and other indirect means. On the other hand, giant liposomes, having diameters ≥ 1 μm, are observable *via* optical microscopy and can be individually isolated and manipulated. In this work, phase-contrast microscopy was used to study adhesion between individual giant liposomes, which was induced by two different methods: 1) Promoting contact between oppositely-charged liposomes; and 2) Injecting aqueous salt solution in the vicinity of liposomes of like composition. In the first method, oppositely charged liposomes were separately prepared and brought into contact. (anionic: palmitoyl-oleoyl-phosphatidylcholine (POPC), cholesterol, and either palmitoyl-oleoyl-phosphatidylglycerol (POPG) or palmitoyl-oleoyl-phosphatidic acid (POPA); cationic: POPC, cholesterol, and didodecyldimethylammonium bromide (DDAB)) The occurrence of adhesion was found to depend upon the mol-% of charged lipid, and adhered liposome complexes proved stable to exposure to a variety of aqueous solutions. Additionally, electrostatic layering of charged membranes was found to occur when adhered oppositely charged liposomes peeled from or collapsed upon each other. In the second method, 0.1 M $CaCl_2$ solution was found to promote adhesion between liposomes of POPC/cholesterol. This adhesion was inhibited upon addition of POPG to POPC/cholesterol liposomes. The results of this study shed light on the nature of adhesion between giant liposomes, and further demonstrate the utility of optical microscopy to evaluate their cytomimetic properties.

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5-50

VESICLES DECORATED WITH MAGNETIC GRAINS N. Kern and B. Fourcade

Phospholipids in solution in water spontaneously form closed « bags » of membranes, resembling biological membranes in size (some microns) and structure (bilayer). We consider a model for vesicles whose membrane has been decorated with small ferromagnetic grains, attached to the bilayer but free to turn. Equilibrium shapes are determined by competition between the bending energy of the bilayer and the magnetic energy of the grains, tending to align the membrane with the applied field. We distinguish two cases :

- A rotating magnetic field, of a frequency of more than a few Hz and a strength of a few millitesla, can induce a shape transition from prolate to oblate ellipsoids. The two regimes are separated by intermediate non-axisymmetric shapes, requiring a second order phase transition.
- A static field applied to vesicles adhering to a substrate forces the vesicle into an ellipsoidal shape, thus allowing it to unbind from the substrate by thermal motion.

In both cases, thermal fluctuations of the vesicle shapes are strongly reduced by the magnetic field.

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5-51

EVIDENCE FOR A SUPERSTRUCTURE OF THE FLUID STATE IN THE LIPID MEMBRANES OF DOPC. B.Klösgen and W.Helfrich.

Fluid lipid membranes of DOPC were investigated by cryo-TEM in their lamellar state. Lipid vesicles were prepared as 2% aqueous suspensions by several methods. Sampling and cryofixation were done at various times within three weeks after vesicle preparation. Sonicated dispersions always consisted of mostly unilamellar vesicles with smooth membranes. Though the fraction of smooth unilamellar vesicles also dominated in the shaken and extruded vesicle dispersions we then abundantly observed objects with anomalous surface features. Among the reproducible appearances were angular vesicle shapes, isolated membrane bends and grainy membrane textures. These superstructures are all features of single membranes. Grainy membranes show up in several modifications as to their density and order. The minimal grain spacing is about 4-6nm. The graininess is not present in small vesicles and in thin tubes. It seems to prefer membrane regions of lower curvature. Smooth and crumpled regions can coexist within one membrane, the domains being separated by a kind of border line. In addition to these appearances of unilamellar vesicles, we also observed objects with numerous passages. We presume them to be formed by membrane fusion of initially enclosed vesicles or as connections of the upper and lower membranes of extended and very flat vesicles. Both the passages and the shapes of smooth vesicles can be described by normal elasticity theory, either as a result of Gaussian or mean curvature bending contributions. The various superstructure appearances must probably be attributed to effects of Gaussian curvature of higher order that allow the system to decrease its total energy. According to such a model, a superstructure of a fluid membrane could manifest itself by a disordered arrangement of local saddles like the textured membranes observed. Single localized hats might produce angular vesicles. Stable folds or deep ridges may result when local saddles cooperatively assemble. The observed features thus require the introduction of non-Hookean terms to describe the equilibrium shapes of fluid lipid membranes.

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5-52

INFLUENCE OF PULMONARY SURFACTANT PROTEINS ON THE PHASE DIAGRAM AND MESOSCOPIC MORPHOLOGY OF PHOSPHOLIPID SURFACE MONOLAYERS.

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Phosphocholines and phosphoglycerols are the main lipid components of the pulmonary surfactant that forms a monolayer separating the air/alveolar interface in mammal lungs. About 50 wt% of the lipid fraction of the surfactant consists of dipalmitoyl-PC, and ~ 5 wt% are peptide or protein. The impact of the bovine pulmonary surfactant proteins, SP-B and SP-C, on isotherms and mesoscopic morphologies in phase separated lipid (DPPC and DPPC/DPPG) monolayers has been studied using fluorescence microscopy. The peptide exerts a marked influence on the lipid's isotherms already at concentrations of 1%. We find that phase separated domains in the lipid/peptide monolayers nucleate in a similar fashion as in the pure lipid monolayers. In contrast, at the termination of the phase transition, where isotherms show an additional discontinuity in compressibility, lipid/peptide surface monolayers are quite distinct from pure lipid. In pure lipid films, a distinction of the ordered and disordered phases as developed during the transition and reported by the partition of a fluorescent label, is retained up to high monolayer pressure. In lipid/peptide films, the ordered domains dissolve at the termination of the phase transition, as indicated by the redistribution of the fluorescent dye. This is probably related to the kink induced by the protein component in the lipid's isotherm. The fast redistribution of the fluorescent label hints at a high self-diffusion coefficient at high monolayer pressure, significantly larger than in the pure lipid monolayer. It seems thus that the protein promotes a softening of the surface monolayer specifically at high surface pressure, corresponding to low surface tension, a state that is relevant to the lung function during breathing.

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5-53

THE RECEPTOR-BINDING AND TRANSLOCATION STEPS OF COLICIN N. Elaine M. Raggett, Graeme Bainbridge, Lynn G. Dover, Alan Cooper*, and Jeremy H. Lakey.

Colicin N is a pore forming toxin produced by *E. coli* which kills other, sensitive *E.coli.* Colicin N requires OmpF as a receptor on its target cell. The structures of both proteins are known to high resolution from X-ray crystallography. The structure of colicin N consists of three separate domains which have the functions of translocation, receptor-binding and pore formation.

All the domains can be produced as separate polypeptides although the translocation domain is unstructured in solution as in the crystal structure. We have used site-directed mutagenesis to map the important sites for the binding to the receptor OmpF, translocation through the membrane and binding to the periplasmic translocator protein TolA. The binding interactions are measured by fluorescence, isothermal titration microcalorimetry and surface plasmon resonance. Results have shown that the TolA recognition box consists of five residues in an unexpected part of the N terminal region. Mutations in the receptor binding domain reveal specific residues required for the recognition of OmpF. Mutagenesis of OmpF has shown that the colicin does not traverse the membrane through the protein's pore. The results provide quantitative biophysical data on the chain of interactions occurring during toxin action.

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5-54

INTERACTION BETWEEN WATER-SOLUBLE MOLECULES AND MEMBRANE: THRESHOLD EFFECT FOR PENETRATION FOLLOWING ADSORPTION. M.F.Lecompte

Interaction of water-soluble molecules (proteins, drugs...) with membrane phospholipids is an interfacial process of general interest in cell biology. Nevertheless, the manner in which they interact at the molecular level, and the relationship of this type of interaction to its function are far from being elucidated.

To answer this question, it was necessary to use membrane models. The validity of two of them was tested and shown to give consistent results. Complementary methods of direct surface measurements on condensed monolayers (electrochemistry and radioactivity) were used, in association with photochemistry on vesicles followed by gel electrophoresis analysis. This approach has been developed in order to study the role of phospholipid components of membranes in the conversion of proenzymes to active enzymes (thrombin) in the course of haemostasis.

It was possible to observe different binding states, with different association constants, and more specifically to detect penetration (and the protein structural domains involved) as distinct from adsorption. The threshold value separating these two steps depends on protein (or drug) concentration, lipid composition and the type of protein (enzyme, substrate, cofactor). There is a balance between electrostatic and hydrophobic interactions, which might reflect different populations of proteins playing different roles in the regulation of such biological processes. Furthermore, structure / function relationships could be established: penetrated proteins are still biologically active.

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5-55

SELF-ASSOCIATION PROCESSES INVOLVING PYRENE LABELED PHOSPHOLIPIDS IN MODEL MEMBRANES. S. Mazères, J-F. Tocanne, and A. Lopez.

Pyrene labeled phospholipids are widely used for investigating the dynamics of lipids in membrane. For a better analysis of data obtained with the steady-state and dynamic fluorescence of pyrene fluorophore attached to phospholipids, we have studied, by means of absorption spectroscopy, phospholipids or glycolipids unilamellar vesicles labeled with pyrene-phosphatidylcholine or pyrene-phosphatidylglycerol. We show that with these probes, a self-association process occurs in the ground state. Pyrene self-association has been characterized and leads to a hypochromic or a hyperchromic effect, depending on the concentration of the Pyrene-probe in the host lipid. The indefinite linear self-association model, in which each association step is characterized by the same equilibrium constant, well describes the data. The equilibrium constant was found to vary between 0.1 M^{-1} and 90 M^{-1}, depending on the pyrene-phospholipid and the host lipid. The consequence of this pyrene-labeled phospholipid self-association, has been interpreted and used for the analysis of the steady-state and dynamic fluorescence of the monomer and excimer excited species of pyrene-labeled phospholipid.

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BOVINE BRAIN CYTOSOLIC 21 kDa PROTEIN: ADSORPTION KINETICS AT THE AIR/WATER INTERFACE AND PENETRATION INTO PHOSPHOLIPID MONOLAYERS. R. Maget-Dana, B. Vallée, D. Lelièvre and F. Schoentgen.

The basic 21 kDa protein was extracted from bovine brain cytosol and its complete amino acid sequence was established: it revealed 50% sequence identity between the C-terminal region of the 21 kDa protein and a central region of the phosphatidylcholine transfer protein. At the present time the biological function of the 21 kDa protein remains partially unclear. However it has been shown to interact with hydrophobic ligands, particularly with phosphatidylethanolamine. The possibility for the 21 kDa protein to be implicated in membrane binding and/or phospholipid transport have been suggested. This would imply an affinity of the protein towards membrane interfaces. C-terminal and N-terminal fragments supposed to be implicated in phospholipid binding were synthesized and their interfacial properties were compared to those of the whole protein. The adsorption parameters at the air/water interface were shown to be affected both by the amino acid concentration in the subphase and by the chain length of the peptide fragments.The ability of the 21 kDa protein to penetrate into phospholipid films was investigated by means of the changes in surface pressure that follow the injection of protein under the film. The penetration of the 21 kDa protein into DMPE films is rather weak and comparable to that observed in the case of DMPC films. The 21 kDa protein penetrates more intensively into DMPG films wich are negatively charged. The surface pressure increase resulting from the protein penetration into various lipid films is lower than that observed in the case of the protein adsorption at the air/water interface. These results suggest that the 21 kDa protein is probably adsorbed to the phospholipid film at the level of the polar heads but does not penetrate greatly into the film.

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MEASURING LATERAL DIFFUSION IN MEMBRANES BY THE STATIONARY FLUORESCENCE OF COPA. **A.U.Acuña, F.Amat-Guerri, C.R. Mateo, E. Quesada and A.A. Souto.**

A simple fluorescence method to quantitate restrictions in lateral mobility in lipid biomembranes has been developed, based on the photodimerization of polyenes in bilayers as described by Morgan et al. (1). The new fluorescent fatty acid *t-octa-decapentaenoic* (*t*-COPA), can be incorporated in substantial amounts into membranes without disrupting the bilayer structure (2). In these conditions, the continuous irradiation (350 nm) of the probe results in a gradual loss of the fluorescence, due to the reactive encounters of COPA molecules. The experimental 2nd order photochemical reaction rate can be interpreted by two competing pathways: one controlled by the lateral diffusion of the probe and an additional "static" term, which is independent of probe mobility. A simple kinetic model allows the separation of the two terms and the accurate measurement of relative changes in the local fluidity of lipid bilayers as a function of temperature, lipid composition, cholesterol content, phase formation, etc.. In addition, values of the lateral diffusion coefficient (D) of *t*-COPA can be determined from these experiments, based on the standard solution of the Smoluchowski diffusion equation for a two-dimension fluid. The fact that the values of D obtained in that way (0.5-1 x $10^{-7}cm^2s^{-1}$, DMPC, liquid-crystal phase) agree closely with those determined by alternative techniques is considered a firm validation of the method proposed here.

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(2).- C.R. Mateo, A.A. Souto, F.Amat-Guerri and A.U. Acuña, *Biophysical J.* 71, 2177 (1996).

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5-58

CALIBRATION OF FLUORESCENCE RESONANCE ENERGY TRANSFER VALUES GAINED ON CELL SURFACES L. Mátyus, P. Salga, A. Bodnár and S. Damjanovich

Different kinds of cell surface receptor clusters have been discovered recently using fluorescence resonance energy transfer (FRET) measurements. This method is capable for identifying molecular interactions, however the exact distances remain obscure, because the classical Förster efficiency-distance relationship is valid only in the case of one donor one acceptor systems. This condition can not be fulfilled when cell surface molecules are labeled with monoclonal antibodies carrying the fluorescent donor and acceptor molecules.

Our aim was to carry out FRET measurements on such cell surface receptors, where the distances are constant, and the only changing parameter is the donor-acceptor ratio of the used labels. For our experiments we used JY B lymphoblastoid cells, and labeled the MHC class I heavy chain with KE-2 or W6/32 monoclonal antibodies and the light chain with L-368 monoclonal antibody tagged with different numbers of donor or acceptor molecules. The FRET efficiencies were measured in a microscope using the photobleaching method. We changed the donor acceptor ratio in a wide range in order to make a suitable calibration curve for other FRET experiments.

The obtained calibration curve gives us the possibility to relate FRET efficiencies to real distances among cell surface receptors.

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5-59

BENDING ELASTICITIES OF MODEL MEMBRANES. TEMPERATURE AND STEROL CONTENT INFLUENCES. P. Méléard, C. Gerbeaud, T. Pott and L. Ferandez-Puente.

Giant liposomes obtained by electroformation and observed by phase contrast video microscopy show spontaneous deformations originating from Brownian motion, similar to that observe during the flickering of erythrocytes. In the case of quasi spherical vesicles, these deformations are quantitatively analyzed using two parameters only, the membrane tension σ and the bending elasticity k_C. At given temperature and membrane composition, σ is changing from one liposome to the other, while k_C is constant as expected for a mechanical material property. Far from the main (thermotropic) temperature transition T_m, k_C is a quadratic function of the chain length for dilauryl phosphatidylcholine (DLPC), dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). k_C is shown to be temperature dependent on approaching T_m, for liposomes containing dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). In the case of DMPC/cholesterol bilayers, we also got evidence for a relation between the bending elasticity and the corresponding temperature/cholesterol molecular ratio phase diagram. Comparing DMPC/cholesterol with DMPC/cholesterol sulfate bilayers at 30°C containing 30% sterol ratio, we show that k_C is independent of the surface charge density of the bilayer. Finally, bending elasticities of red blood cell total lipid extracts lead to a very low k_C at 37°C if we refer to DMPC/cholesterol bilayers and to an unexpected behavior at 25°C, probably related to phase coexistence within the protein free membrane.

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5-60

CYTOPLASMIC LOOP 6-7 BINDS CA^{2+} AND IS FUNCTIONNALLY IMPORTANT FOR THE SARCOPLASMIC RETICULUM CA^{2+}-ATPASE. T. Menguy, P. Falson, F. Corre, L. Bouneau, A. Gomez de Gracia, S. Soulié, F. Centeno, J.V. Moller, P. Champeil, and M. le Maire.

Limited proteolysis of the rabbit SERCA 1 Ca^{2+}-ATPase by proteinase K generates a number of fragments which have been identified previously. Here, we have focused on a C-terminal peptide of 20 kDa, p20c, starting at G808. This peptide binds Ca^{2+} as deduced both from the change of its migration rate when running in SDS-PAGE in the presence of Ca^{2+}, and from the fact that is also labelled in $^{45}Ca^{2+}$ overlay experiments. In contrast, p19c, a proteolysis fragment identical to p20c but for 10 amino acids missing at the N-terminal side, does not bind Ca^{2+}. Two cluster mutants in this region, D813A-D818A and D813A-D815A-D818A, expressed in the yeast *Saccharomyces cerevisiae,* had a very low Ca^{2+}-ATPase activity and were unable to form a phosphoenzyme from ATP up to 1 mM Ca^{2+}. Furthermore, the mutant enzymes were phosphorylated by inorganic phosphate, even in the presence of Ca^{2+}, which inhibits phosphorylation of the wild type enzyme. Thus the Ca^{2+} binding region 808-818 is functionnally important for the enzyme, as found recently for the analogous H^+,K^+ ATPase (Swarts, H.G.P., Klaassen, C.H.W., de Boer, M., Fransen, J.A.M., and De Pont, J.J.H.H.M. (1996) *J.Biol.Chem.* 271, 29764-29772). It is remarkable that, in the Ca^{2+}-ATPase, these amino-acids are located just after the putative transmembrane segment M6, in the 6-7 cytoplasmic loop.

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5-61

INFLUENCE OF AN INCLUSION COMPLEX ON THE CHARGE-TRANSFER RESISTANCE OF A CHEMISORBED CYCLODEXTRINE MONOLAYER. A. Michalke, A. Janshoff, C. Henke, M. Sieber and H.-J. Galla.

Purpose: One of the most interesting properties of cyclodextrines is their ability to incorporate many anorganic or organic, ionic or non-ionic guest molecules into their cavity. Based on synthesized cyclodextrine derivatives, we developed a sensitive device for the detection of small hydrophobic ions.

Methods: Monomolecular layers of 3-mercapto-propane-(N-mono-6-deoxy-β-cyclodextrin)-amide (MPA-CD) were attached to gold substrates by means of self-assembly techniques and characterized by impedance spectroscopy using the electroactive markers $[Fe(CN)_6]^{3-}$ / $[Fe(CN)_6]^{4-}$. 1-Aminoadamantane hydro-chloride and 1-adamantanecarboxylic acid were used as guest molecules.

Results: The influence of the specifically adsorbed and differently charged guest molecules on the charge-transfer resistance was demostrated by the repulsion and the attraction of the electroactives markers $[Fe(CN)_6]^{3-}$ / $[Fe(CN)_6]^{4-}$. Moreover, the dependence of the charge-transfer resistance on the concentration of the guest molecule was registered. Treating the monolayers with a solution of pure cyclodextrines seems to remove most of the adsorbed molecules.

Conclusions: Further investigations will show, whether MPA-CD-monolayers can be established as useful and renewable sensing devices for certain guest molecules.

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5-62

THE BINDING OF BOVINE SERUM ALBUMIN (BSA) TO LIPOSOMES. A. Michnik.

Proteins interact with the lipid bilayer by binding or adsorption to its surface, insertion into the hydrocarbon region of the membrane interior, or penetration through the membrane. The binding of BSA to egg yolk lecithin - cholesterol (molar ratio 2:1) liposomes was studied as a function of protein concentration and membrane charge (octadecylamine put in a positive charge and dihexadecyl hydrogen phosphate - a negative charge). Complexes prepared by the three methods ({1} BSA was added to vesicles during their production or {2} ready liposomal solution was only mixed or {3} additionally incubated with BSA at room temperature) were isolated by gel filtration on Sepharose 2B-Cl columns using 0,05 M TRIS HCl buffer (pH=7,2) as the eluant. Protein elution was monitored by absorbance at 280 nm and by the technique of Lowery et al.. The positively charged vesicles were retained on the Sepharose 2B-Cl gel, thus the determination of protein content in liposomal fractions was unable for this kind of liposomes. In this case the amount of binding protein was estimated by the difference between the amount of BSA in the tested sample and the summary amount determined in all protein fractions. The quantitative results showed that the amount of BSA bound to the liposomes was the greatest one in the case of {1} - st complex preparing method (above 20 % of BSA amount originally present in solution) and for negatively charged liposomes. The process of incubation increased the protein binding to the uncharged and positively charged vesicles and decreased the amount of BSA bound to the negatively charged liposomes. The dependence of the protein binding on membrane charge indicated that electrostatic attraction plays a part in the lipid - protein interaction.

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5-63

D-GLUCOSE UPTAKE IN HUMAN LYMPHOCYTES MODULATED BY SOME SPECIFIC LIGANDS AND CHEMICAL AGENTS

N. MOISOI, I. PETCU

The aim of this work is to observe the modifications induced in D-Glucose uptake by the activation of some membrane receptors, by the variation of the membrane fluidity and by heat shock. The measurements were performed on human lymphocytes (10^6 cells/ml) after the following treatments: heat shock (30 min., 45° C), insulin (9 μM, 1 h), concanavalin A (50μM, 15 min., 30 min., 5 h), phytohemagglutinin A (50μM, 5 h), diamide(2.5μM), ethanol (1%, 2%), butanol (2%). The insulin presence and the heat shock are inducing a significant increase of the glucose uptake. The mitogen agents Con A and PHA induce observable increase in the sugar uptake only after long term incubations. The subsequent treatment with insulin does not produce an additional modification of this process, the uptake preserving approximately the same extent. The other chemicals modify the glucose transport showing a decrease (diamide treatment) or an increase (alcohol treatment) of the process probably in correlation with the changes induced in the membrane fluidity. The results show that heat shock, insulin and mitogen stimulation are inducing similar increases in hexose uptake. This observation might reflect analogous modifications of plasma membrane function for all the investigated situations. The mechanism proposed in the literature, to explain the increase in sugar uptake after insulin or stress treatments, assumes that a reversible and specific translocation of the glucose transporter protein take place, from an inactive, intracellular site towards the plasma membrane. The absence of a supplementary increase in the sugar transport for mitogen stimulated cells after the insulin addition could prove that each specific ligand (insulin, Con A, PHA) is inducing the translocation of all the glucose transporters to the plasma membrane. The membrane fluidity, a parameter that was modified by chemical treatments, seems to have a strong influence on the glucose uptake and on its modulation by the insulin receptor activation.

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5-64

ACTIVATION OF THE COMPLEMENT SYSTEM BY PLANAR ASYMMETRIC GLYCOLIPID/PHOSPHOLIPID BILAYERS. M. Münstermann, A. Wiese, K. Brandenburg, U. Zähringer, and U. Seydel

As major amphiphilic constituent, Gram-negative bacteria express on the surface of their outer membrane a glycolipid. This is in most cases a lipopolysaccharide, LPS, in some a glycosphingolipid, GSL (e.g., *Sphingomonas paucimobilis*). We have elucidated the possible complement activation as an important part of the immune response to invading bacteria and the activation pathway for rough mutant LPS (Re and Rd_2 from *Salmonella minnesota* R595 and R4, respectively) and the GSL fraction with the shortest sugar moiety, GSL-1. To study the influence of charges, we prepared derivatives of GSL-1, in which the carboxyl group was methylated or reduced.

A planar reconstitution model of the lipid matrix of the outer membrane was used to detect the induction of complement pores formed by the assembly of C5b-9 membrane attack complexes. The lipid matrices were reconstituted as asymmetric bilayers according to the Montal-Mueller method with LPS or GSL on one and a mixture of phospholipids (PL) on the other side.

After addition of whole human serum to the glycolipid side, the membranes were destabilized, and an increasing membrane conductance was observed. At the beginning of the conductance trace, the incorporation of C9 monomers was reflected by a stepwise increase of the conductance. In the cases of LPS Re and GSL-1, the single-step amplitudes and the pore geometries were similar: Both, circular pores and "leaky patches" (linear, not completely C9-aligned lesions) could be observed. In order to elucidate the activation pathways, we used sera depleted in C1q or Ca^{2+}, respectively. We found that LPS Re and LPS Rd_2 activated the classical pathway. GSL-1 activated the alternative pathway, whereas the derivative of GSL-1, in which the carboxyl group was reduced, activated the classical pathway.

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5-65

FLUORESCENCE SPECTROSCOPY AS A TOOL TO ANALYZE STRUCTURAL ORGANIZATION OF COSUBSTRATE BINDING SITES IN THE E. COLI NA^+/SUGAR TRANSPORTER. I. Mus-Veteau, C. Maehrel and G. Leblanc.

To assess the individual contribution of N- or C-terminal domains of the permease to the fluorescence variations induced by the coupling ion and sugar binding, we replaced the two tryptophans located in its C-terminal half (W299 and W342) by a phenylalanine. Persistence of the ion-induced signal quenching in a permease carrying only the six other tryptophans of the N-terminal domain is consistent with previous suggestion that this domain accommodates the ion-binding site. Concerning the sugar, while α-galactosides increase essentially the fluorescence of W299 and W342, β-galactosides enhance the signal of W299 and of one (or more) of the N-terminal tryptophans but quenche that of W342. Moreover, studies realized using a fluorescent sugar (dansyl galactoside) show an energy transfer between Trp and sugar which is partly inhibited when the W 299 and W 342 are replaced by phenylalanine. All these data suggest that W299 and W342 are at or close to the sugar binding site and that this latter is lined by the C-terminal helices IX and X. Moreover, as N-terminal tryptophans are also pertubed by binding of sugars with β configuration and transfer their energy to a fluorescent form of this sugars, it is suggested that one (or more) helix of the N-terminal half may be also at or near the sugar binding site. This implies close proximity and/or tight functionnal linkage between some N-terminal helices and helices IX and X of the C-terminal domain of the transporter.

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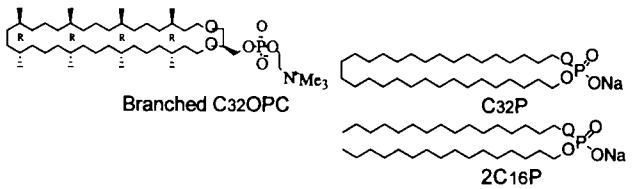
5-66

MEMBRANE PROPERTIES OF ARCHAEBACTERIAL MACROCYCLIC LIPIDS. Y. Nakatani, O. Dannenmuller, K. Taguchi, T. Eguchi and G. Ourisson

The most striking structural feature of archaebacterial lipids is the macrocyclic ring structures. We are interested in the physico-chemical properties of these unusual lipids.



First, to analyze the consequence of the C-C linkage at the alkyl chain termini, we compared the properties of a macrocyclic phosphate (C32P) and dihexadecyl phosphate (2C16P) using FT-IR, fluorescence anisotropy and monolayer π-A isotherm measurements. We have found that the ring closure raises the phase transition temperature (Tm) and reduces the ratio of the gauche conformation which consequently decreases the mobility of the lipid chain above the Tm.

Then, using a video-enhanced optical microscope and a confocal one, we have observed different types of self-organized structures, obtained from a water suspension of a macrocyclic archaebacterial lipid (Branched C32PC). For example, we have observed uni- or multi-lamellar vesicles, tubules and oligo-vesiculer vesicles. By use of Nile red, a hydrophobic fluorescent probe, we could also observe various changes between the different morphological structures.

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5-67

COMPARED EFFECTS OF A SERIES OF DIHYDROPYRIDINES ON THE *P*-GLYCOPROTEIN ATPase STIMULATION BY VARIOUS DRUGS. C. Pascaud, M. Garrigos and S. Orlowski.

P-glycoprotein (P-gp) is the plasma membrane protein responsible for the multi-drug resistance (MDR) phenotype. It is believed to efflux various hydrophobic drugs out of the MDR cells at the expense of MgATP hydrolysis. We aimed to investigate the broad specificity of P-gp transport substrate recognition by characterizing the mutual relationships between various modulators (cytotoxic drugs or MDR-reversing agents) of its ATPase activity. We used native membrane vesicles prepared from highly resistant MDR cells (DC-3F/ADX, derived from the Chinese hamster lung fibroblasts DC-3F), in which P-gp represents c.a. 15% of total membrane proteins. We tested the respective effects on P-gp ATPase of nicardipine, nimodipine, nitrendipine and nifedipine, four dihydropyridine (DHP) derivatives having a decreasing hydrophobicity. Only nicardipine is a clear activator ($K_{1/2}$= 0.6 μM, activation factor 2.1 with respect to the basal activity), and this activation is competitively inhibited by vinblastine in the micromolar range. When assayed in the presence of verapamil, another P-gp ATPase activator, nicardipine becomes inhibitor, which demonstrates the binding of both molecules on P-gp forming a ternary complex. The three other DHPs all inhibit verapamil-dependent P-gp ATPase stimulation, but at higher concentrations than nicardipine does. The tested DHPs also inhibit progesterone-dependent P-gp ATPase stimulation, for the same concentration ranges than for the inhibiting effect on the verapamil-dependent stimulation. As a whole, DHPs, verapamil and progesterone appear as non-competitive modulators of P-gp ATPase activity, thus probably defining different binding sites on P-gp. In contrast, nifedipine inhibits the nicardipine-dependent P-gp ATPase stimulation at higher concentrations than it inhibits the verapamil-dependent P-gp ATPase stimulation.

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5-68

THE EFFECT OF HE-NE LASER BEAM ON THE METABOLIC ACTIVITY IN EGGS OF SCHISTOCERCA GREGARIA (L).

A.M. El-Gindi[1], W.G. Osiris[2] and N. El-Kes[3].

Eggs of *Schistocerca gregaria (L)* were exposed to He-Ne laser beam at four exposure periods to study the variations occured in : GOT, GPT; acid and alkaline phosphatase activities; total protein and albumin contents. The effects of He-Ne laser beam at two exposure periods on the cholesterol; RNA, DNA contents and on the ionic elements (Na^+, K^+, Ca^{++}) were also investigated. The data showed that measurable changes were detected after exposure to the different exposure times up to 120 minutes in comparison with the control values. Significant decrease in GOT (≈ 36%) in eggs exposed to 120 minutes, were detected. Very high significant increases in acid and alkaline phosphatase (≈ 153% and 114 %, respectively) after exposure for 60 minutes, were determined. No remarkable change in the protein content while significant increase in the albumin content (≈ 47.2%) were obtained. Significant decrease in cholesterol content (≈ 23%) was detected. Very high significant changes in RNA (increase ≈ 98%) and DNA (decrease ≈ 63%) were measured. Moreover, measurable and remarkable significant changes in the ionic contents due to laser-irradiation at the 15 and 30 minutes exposure times were calculated.

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5-69

A DETAILED STUDY OF THE FLUORESCENT LIPIDIC PROBE LAURDAN M. Viard, J. Gallay, M. Vincent, B. Robert and M. Paternostre

The amphiphilic fluorescent probe Laurdan has been used to visualised lipidic phase transition and the vesicle to micelle transition of mixed amphiphilic systems. Beacause the origins of its high sensitivity to the environement are still unclear a detailed study of the probe has been done. IR and Raman measurements show that the probe is plane in its fundamental state whatever the solvent used. Absorption spectra demonstrate however, an heterogeneity of the absorption transitions. Detailed steady state and time resolved fluorescence studies have been done to provide information about the different reactions occurring at the excited state of the molecule. The desexcitation pathway of Laurdan is complex and revealed two major reactions at the excited state. The MEM analysis of the fluorescence decays evidenced a very fast component which has been attributed to an intramolecular reaction and a slower one attributed to the solvent relaxation process. Thereafter, the probe has been incorporated in different types of mixed aggregates of controlled composition, i.e. pure liposomes, mixed micelles and pure micelles. Steady state and time resolved fluorescence measurements have been performed. Going through the transition induced an acceleration of the reactions at the excited state and a decrease of the fluorescence lifetime of the probe.

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5-70

MEMBRANE FUSION INDUCED BY A SHORT SYNTHETIC PEPTIDE COVALENTLY BOUND TO LIPOSOME LIPIDS. **E.I.Pécheur**, Hoekstra D., Sainte-Marie J., Maurin L., Bienvenüe A. & Philippot J.R.

The fusogenic properties of an amphipathic net-negative peptide (wae 11), consisting of 11 amino acid residues, were studied. We demonstrate that whereas the free peptide displays no significant fusion activity, membrane fusion is strongly promoted when the peptide is anchored to a liposomal membrane. The fusion activity of the peptide appears to be independent of pH, and membrane merging is an essentially non-leaky process. Vesicle aggregation is a prerequisite for fusion to occur. For this process to take place, the target membranes required a positive charge which was provided by incorporating lysine-coupled phosphatidylethanolamine (PElys). By monitoring changes in intrinsic Trp fluorescence, in conjunction with KI-quenching studies, it would appear that hydrophobic interactions facilitate the fusion event, possibly involving (partial) peptide penetration. Such a penetration may be needed to trigger formation of a transient, non-bilayer structure. Since lysophosphatidylcholine inhibited, while monoolein strongly stimulated peptide-induced fusion, our data indicate that wae 11-induced fusion proceeds according to a model consistent with the stalk-pore hypothesis for membrane fusion. By monitoring lipid mixing between peptide-coupled liposomes and target vesicles of defined lipidic composition, it is found that fusion increases with temperature, displaying a threshold around 15°C, while aggregation remains unaffected over a 4 to 40°C-temperature range. By measuring the self-quenching of the fluorescent analog of the peptide carrier molecule, we demonstrated that peptide and PElys clustering were related to and commensurated with the temperature-dependent fusion of the two membranes.

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5-71

FILIPIN-ERGOSTEROL INTERACTION IN LIPID VESICLES. A PHOTOPHYSICAL STUDY. L. Loura, M. Castanho, A. Fedorov and M. Prieto

Filipin, a macrolide antibiotic, is known to interact selectively with ergosterol, a constituent of fungi membranes.

In this work, a fluorescent analogue of ergosterol, dehydroergosterol (DHE), was used and its energy transfer efficiency to filipin (R_0 = 26.4 Å) was measured in a model system of membranes (small unilamellar vesicles (SUV) of dipalmitoylphosphatidylcholine (DPPC) at 25°C) to obtain information on the filipin-sterol interaction.

A model was developed and fitted to both time-resolved and steady-state fluorescence data of DHE in the presence of filipin.

The results point to the formation of both filipin-filipin aggregates (evidence from: i) the non-linear dependence of time-resolved energy transfer on acceptor concentration, a correction factor for dimerization being needed to account for the results; ii) spectral changes of filipin absorption in DPPC SUV, with enhancement of the λ = 325 nm peak (excitonic absorption, suggesting "stack" geometry for the dimer); and iii) filipin fluorescence self-quenching) and DHE-filipin aggregates (evidence from static quenching of DHE fluorescence by filipin). The model is best fitted by aggregation constants' values of K_1 = [DHE-filipin]/[filipin] = 0.67 at DHE:DPPC = 0.01, K_2 = [filipin-filipin]/[filipin]^2 = 6.1×10^5 M^{-1} (concentrations in mol/volume of lipid phase).

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5-72

"PULSE FIRST" ELECTROFUSION OF EUCARYOTIC CELLS. C. Ramos and J. Teissié.

Cell fusion can be obtained by application of transient electric pulses on cells previously brought in close contact (electrically induced fusion or electrofusion). In 1986, a new approach has been decribed, the "pulse first contact" (PF) method. Cells are electropulsed and then brought in close contact. The PF electrofusion is not directly created by the field but is due to the existence of a peculiar state of the electropermeabilized plasma membrane. The relation between electropermeabilization and electrofusion of animal cells was studied. We show that when two permeabilized membrane areas are in close contact, fusion occurs. The fusion yield increases when the fraction of cell surface altered by electric field and when the efficiency of electropermeabilization controled by the number and the duration of pulses are high. This supports a molecular description of electropermeabilization, where a large number of small defects is present affecting the membrane / solution interface to obtain a fusogenic state.

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5-73

COMPARATIVE STUDIES OF THE INTERACTION OF BASIC PEPTIDES WITH ACIDIC PHOSPHOLIPIDS. M. Requero and A. Blume.

A considerable part of the protein component of biological membranes is only loosely associated with the membrane, mainly via electrostatic interactions with either lipids or other membrane proteins. Thus in order to gain a new insight in the characterization of the nature of these interactions, we have studied the interaction of basic peptides, namely di-, tri- tetra- and pentalysine, with sonicated vesicles of acidic phospholipids (DMPG) using the differential scanning (DSC) and the isothermal titration calorimetry (ITC). The former technique characterize the effect of the basic peptides on the phospolipid phase transitions. While the latter technique allows to measure directly the heat absorbed or evolved during molecular associations. By directly observing the heat (enthalpy) of a reaction, ITC provides a model-independent measure of $\Delta H°$ and allows the determination of the heat capacity change, ΔCp, from experiments conducted at two or more temperatures.

The nature of association of the basic di-, tri-, tetra- and pentapeptide with the SUVs is clearly different. While Lys_2 and Lys_4 interact with DMPG vesicles with a small heat change, the interaction of both Lys_3 ,and Lys_5 yield observable higher values. These effects are also reflected in differences in the phase transition behaviour after the addition of Lys_n . Lys_2 and Lys_4 affect only slightly the phase transition temperature (T_m) of DMPG, whereas Lys_3 and Lys_5 induce clearly a T_m shift and a decrease in the enthalpy values.

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5-74

MAMMALIAN CELL PERMEABILIZATION TO MACROMOLECULES BY ELECTRIC FIELD. EVIDENCE FOR A MACROPINOCYTOSIS LIKE PROCESS. M.-P. Rols, C. Delteil, M. Golzio and J. Teissié.

Application of high electric field pulses to cells can lead to the transient permeabilization of their plasma membranes. Electropermeabilization to small molecules is a three step process of induction, expansion of transient permeated structures that occurs during application of the electric pulses (ms time range), and then annihilation, a phenomenon that depends on the temperature and on the cell cytoskeleton (min time range). A free diffusion across the membrane takes place after the pulse as long as the resealing is not complete. No exchange is observed after the resealing.

Electrotransfer of macromolecules appears not to obey the same law. DNA penetration into cells is not only an electrically mediated process but is a cell mediated one. Macromolecules such as fluorescent dextrans (FD-70) and enzymes (β-galactosidase) can enter the cells only when present during pulsation. An homogeneous distribution of the transfered molecules is detected in the cytoplasm. They are observed to penetrate into the cells when added up to one hour after the electric field application, when penetration of small molecules is no more present. In that case, they are found into cytoplasmic macrovesicles and not homogeneously distributed into the cytoplasm. This phenomenon is inhibited when cells are pretreated with drugs affecting the cytoskeleton. Long term effects of electropermeabilization are induced at the cell level.

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5-75

INFLUENCE OF ACYL CHAIN LENGTH ON BINDING PROPERTIES OF LIPIDATED PROTEINS. M-O. Roy, J-C. Mani, M. Boyer, J. Chopineau and M. Jullien.

Many proteins are covalently modified by lipidation. These acylated proteins, which include heterotrimeric and small G proteins, tyrosine kinases and viral proteins, possess one or two lipids of different types containing 14 to 20 carbon atoms. These modifications are thought to allow otherwise soluble proteins to associate with membranes. The issue that has been addressed concerns the overall strength of the hydrophobic interactions involved in this membrane attachment and its dependence on the fatty acyl chain length.

In order to account for the effect of acyl chain length on protein-lipid interactions, independently of other kinds of interactions, a system model has been chosen. Bovine pancreatic ribonuclease A has been chemically acylated on its N-terminal lysine, using reverse micelles as microreactors. The interaction of the modified protein with phospholipids has been monitored by surface plasmon resonance analysis. The binding affinity for lipids has been found to increase by one order of magnitude per CH_2 group added.

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5-76

HYDRATION, DYNAMICS AND FUNCTION OF PHOTOSYNTHETIC MEMBRANES. S.I.Aksyonov, P.P.Knox and A.B.Rubin

Dynamics of the protein component, the polar and non-polar regions of lipids in three species of photosynthetic membranes of purple bacteria *R. rubrum, Rb. sphaeroides* and *E. shaposhnikovii* was studied under various relative humidities by ^1H, ^{13}C and ^{31}P NMR spectroscopy. The results were compared with functional and other characteristics of these membranes and also with data for model systems. Analysis of these data allows to discriminate four stages of hydration which associated with specific changes in structure, dynamics and function of photosynthetic membranes. The hydration of some polar groups at the first stage leads to local changes in the dynamics of the protein component that influences the recombination of photoactive pigment P and intermediate acceptor Q_A . The second stage is induced by incorporating water molecules into hydrogen bonds between the polar head groups of the lipid phase and within macromolecules that results in changes of dynamics of membranes, efficiency of the electron transfer between the quinones, efficiency of photooxidation of cytochrome c. In the third stage all polar groups are hydrated because of the appearance of free water with a high values of dielectric constant. This makes possible lateral mobility of membrane component and regulation of photosynthetic processes mediated by mobile carriers and distance variation between the interacting macromolecular components. Finally, at a high water content conditions for regulating the processes of photosynthesis at the cell level are realized. The mechanisms influencing these processes and the efficiency of regulation of the electron transfer processes in various parts of the photosynthetic chain are discussed.

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5-77

NMR AND NEUTRON DIFFRACTION ANALYSES OF ORIENTED PHOSPHOLIPID BILAYER INTERACTING WITH ANNEXIN V. O. Saurel, P. Demange, V. Gordeliy, E. Amalric-Bellet, and A. Milon.

Structural investigation of protein-membrane interactions is fundamental to understand many biological functions. We used complementary methods; solid state NMR and neutron diffraction experiments to obtain structural and dynamic informations both on phospholipids and proteins. The most efficient membrane model is the oriented sample, single orientation of phospholipids increases the resolution and the signal to noise ratio as compared with liposomes. On the other hand neutron diffraction experiments on oriented bilayers allow us to determine the Fourier profile of the bilayer.

We are especially interested in Annexin V (35 KDa), which binds to acidic phospholipids in the presence of calcium ions. We have prepared highly oriented bilayers composed of PC/PS (9:1), calcium, HEPES and annexin V, and checked the degree of orientation using ^{31}P NMR. This preparation has permitted to perform neutron diffraction experiments on D16-ILL instrument, to obtain 4 diffraction orders and to determine the water distribution profile. Furthermore ^{31}P NMR and ^{2}H NMR performed on deuterated-PC sample ($P(d_{31})OPC$ and $DMPC(\alpha\beta d_4)$) confirmed results previously obtained on large vesicles: 1) Annexin V did not modify the orientation and the dynamics of phospholipids both on the polar head group and on the hydrophobic chains. 2) The interaction of the protein decreased significantly T_{2e} of PC without affecting T_1 both on vesicles and oriented samples. This differential effect on T_1 and T_{2e} reveals that annexin V-lipids interaction induces slow motions on phospholipids. Two motions could explain these results : slow reorientation of the lipid molecules near and/or under the protein and chemical exchange between «bound» and «free» lipids.

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5-78

NEURONAL GROWTH CONE COUPLES TO TRANSISTOR.

R. Schätzthauer, P. Fromherz.

Neuron-silicon junctions were obtained hitherto by the attachment of cell bodies to silicon microstructures [1, 2]. In those systems the membrane was bound to the chip by poly-lysine; the membrane in the junction had ohmic properties. It is an open question (i) whether spontaneous outgrowth of neurons can form contacts at all and (ii) whether the intrinsic nonlinear features of neuronal membranes are visible in such contacts. We studied the outgrowth of Retzius cells from the leech on chips coated with concanavalin A. Extended growth cones were formed. We stimulated the cell body by an impaled microelectrode and observed the response of transistors which were located at a distance from the cell body beneath the growth cone. In some cases we observed negative voltage transients on the gate induced by action potentials. This response is opposite to the effect expected for linear coupling. It is not known at this moment, which ionic currents are involved.

[1] P.Fromherz et. al., Science 252 (1991) 1290.
[2] P.Fromherz, A.Stett, Phys.Rev.Lett. 75 (1995) 1670.

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5-79

STRUCTURAL ANALYSIS OF THE DETERGENT INDUCED FILAMENT FORMATION IN THE PHOSPHATIDYLGLYCEROL-CHOLESTEROL-CHAPS MODEL BILE SYSTEM. B. Klösgen[1], Th. Schröder[2] and Th. Schürholz[2].

Bile detergents induce the formation of cholesterol (Ch) crystals from mixed Ch-phospholipid vesicles, when the Ch content is [Ch] > 30%. Large filaments and helical ribbons are observed as intermediate structures in the process of crystallization. These structures dominate for days or weeks until all fibers are transformed into monohydrate crystals. In the system phosphatidylglycerol/cholesterol/CHAPS the maximum fiber formation occurs at $X_{CHAPS} \approx 0.93$. The yield of fibers is about 100 × higher in the presence of phosphatidylglycerol than with phosphatidylcholine. The high yield is useful for structure determination. Screening of negative charges by high ionic strength prevents the formation of the filamentous transition structures (Schröder, T. and Schürholz, T., 1996, *Eur. Biophys. J.* 25:67-73). The kinetic and the structural changes during the microstructure formation were analyzed by turbidity measurements, electron microscopy and darkfield microscopy.

Three kinetic phases could be distinguished by turbidity measurements during the first 16 h of fiber formation: (i) A fast solubilization of the vesicles with a half-life of 0.5 min. (ii) An intermediate period of about 2 hours of apparently no change in the particle size and (iii) A strong increase in OD indicating the beginning of filament formation.Cryo-TEM pictures revealed vesicles with one or two bilayers ≈ 30 min after detergent addition (phase ii). Samples taken after 14 h (phase iii) show long thin fibers that may extend over several µm. The fibers seem to consist of ribbons that are each about 50 nm wide. The ribbons exhibited series of strictures with a repeat distance of ≈ 300 nm. Here the filaments had the highest contrast, showed about 5-6 layers and were probably seen in the cross-section view every 180° repeat. In the darkfield microscope the first filaments were seen after 16 h. These fibers then had a length of several 100 µm. The cryo-TEM results prove that also the thin filaments are helical ribbons, yet not resolved in the light microscope and possibly concealed in the negative stain technique. Pictures taken earlier at about 4 h after vesicle solubilization already exhibited very thin unilamellar filaments. It is supposed that these layers mostly consist of cholesterol with the phospholipid and CHAPS serving to stabilize the hydrophobic border. The helical pitch seems to increase as the number of layers increases until the large helices (Ø = 1-20 µm) are observable by the light microscope. No aggregated vesicles as intermediate structures were ever observed.

We therefore suppose that the lamella growth proceeds by gradual apposition of cholesterol monomers, and not by vesicle fusion, though partially dissolved vesicles may serve as nuclei for Ch-filaments.

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5-80

EFFECTS OF TEMPERATURE ON ANTIBIOTIC-INDUCED CONDUCTANCE IN MUSCLE CELL MEMBRANE. N.Shvinka.

Potassium and rubidium conductance induced in muscle cell membrane by gramicidin A, head to head covalently linked gramicidin dimer, amphotericin B and nystatin was investigated. Conductance was measured on single frog skeletal muscle fibers under current-clump conditions using a double sucrose-gap technique. The temperature effect on conductance was measured with a thermistor (0.4 mV/°C) placed in the test gap of the chamber.

The increase of temperature by 8-10°C resulted in a considerable rise of both gramicidin- and gramicidin dimer- induced conductance due to the formation of new gramicidin channels. The effect was much greater than in the case of bilayers indicating a remarkable entropy change in the muscle fibre membrane. The temperature dependence of adsorption was more pronounced than that of desorption. The temperature-dependent free energy of channel formation $\left(\Delta G - T\frac{d\Delta G}{dT}\right)$ was in the order of +30...+50 kcal/mol.

The temperature dependent increase of potassium conductance gramicidin-induced channels and in natural potassium channels (inward rectifier) was nearly the same.

The decrease of amphotericin- and nystatin- induced conductance with temperature rise was observed. The decrease in the steady-state polyene- induced conductance with temperature was caused both by an increase of the destruction rate constant and a decrease in the formation rate constant, the letter being the predominant effect. The observed decrease of the formation rate seams to depend on the melting of the aggregates from which channels are formed.

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5-81

ON THE INTERACTION OF LUNG SURFACTANT PROTEIN SP-C WITH PHOSPHOLIPIDS: A TOF-SIMS STUDY N. Bourdos, O. Brox, F. Kollmer, H. Rulle, A. Benninghoven, H.J. Galla and M. Sieber

The lung surfactant is a complex lipid/protein mixture reducing the surface tension of the alveoli. The saturated phospholipids dipalmitoylphosphatidylcholine (DPPC) and negatively charged phosphatidylglycerols like DPPG are two of the surfactant's main lipid components. As one of the four surfactant-associated proteins, the small hydrophobic SP-C is considered to play a major role in the unique respreading properties of the monolayer formed at the alveolar air/water interface during respiration. A model system containing DPPC, DPPG, and SP-C was studied to explore the mechansims underlying these properties. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) was used to directly visualise domain structures of demixed phases in a monolayer. This method has the advantage to detect molecules like lipids and small proteins laterally resolved. Prior to detection, the monolayer was transferred via the LB technique to a conducting substrate to make it accessible to TOF-SIMS investigation. A dye (NBDPC) was added for additional visualisation by fluorescence microscopy. Integrated positive ion mass spectra of the pure substances DPPC, DPPG, and SP-C revealed peaks directly related to the protonated intact molecule ions (the SP-C spectrum revealed a peak at m/u = 4023), as well as fragment ions due to cleavage in the low mass range. Along with the „complete" mixture DPPC/DPPG/SP-C, binary mixtures such as DPPC/DPPG or DPPC/SP-C were studied. In all the mixed systems peaks related to the intact molecules, characteristic fragments, or substrate-associated ions, were observed, and imaging at a high lateral resolution showed typical domain structures of demixed phases. TOF-SIMS is well suited to analyse the chemical composition and to image spatial structures of biologically relevant monomolecular systems.

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5-82

SPECTROSCOPIC STUDIES OF THE INTERACTION OF Ca^{2+}-ATPase-PEPTIDES WITH DODECYLMALTOSIDE AND BROMINATED ANALOGS. S. Soulié, B. de Foresta, G.B. Bloomberg, J.D. Groves, and M. le Maire.

It has been predicted that the sarcoplasmic reticulum Ca^{2+}-ATPase is structured in 10 transmembrane segments (M1 to M10). Most of the 13 Trp residues are believed to be localized in the membrane, near to the lipid-water interface. We have studied the position of Trp 832, using two synthetic peptides: the fragment 808-847 corresponding to the loop 6-7 and the segment M7, and the fragment 818-847, which has a shorter hydrophilic loop. We have followed by fluorimetry their interaction with detergents using two brominated analogs of dodecylmaltoside (DM): 7,8-dibromododecyl-β-maltoside (BrDM), and 10,11-dibromoundecanoyl-β-maltoside (BrUM). Through their bromine atoms, such detergents quench the fluorescence of the Trp with which they are in contact (B. de Foresta et al., Eur. J. Biochem. 241, 343-354, [1996]). The curves of fluorescence quenching of Trp versus the concentration of brominated detergent show that the formation of peptide-detergent complexes is completed for both peptides at a detergent concentration close to the CMC. Fluorescence emission spectra of the peptides are similar in DM, or in brominated analogs. A similar change in fluorescence intensity was observed with both peptides in mixtures of brominated and non-brominated detergents at various molar ratios, indicating that they are inserted similarly in the micelles. With BrDM, the residual fluorescence is about 37%, while for a model compound, tryptophan octyl ester, it is about 3%. This difference suggests that Trp 832 is located near the micellar interface. In the presence of BrUM, the residual fluorescence is slightly lower (27%) which may reflect the greater flexibility of the distal end of the hydrophobic chain of this detergent. These data will be correlated to analyses of these peptides by circular dichroism in SDS and/or DM micelles in order to provide information on their secondary structure.

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5-83

YEAST CELLS PHOTOSENSITIVITY DEPENDS ON THE TYPE OF 5-AMINOLEVULINIC ACID-INDUCED PORPHYRINS. M. G. Strakhovskaya, G. Ya. Fraikin and A. B. Rubin

Visible light irradiation inactivates yeast cells without exogenous sensitizers due to the formation of endogenous membrane-bound porphyrin compound and mitochondrial porphyrins. The accumulation of 5-aminolevulinic acid-induced different types of endogenous porphyrins and the relative sensitivities of yeasts Candida guilliermondii to visible light (400-600 nm) are described now. The porphyrin formation was studied by means of spectrofluorometric analysis of dense cell suspensions in the triangular cuvette and ether extracts from the cells. When exposed to adequate concentrations about 0,1-0,2 mM of 5-aminolevulinic acid (ALA) yeast cells are slightly sensitized to visible light. Being used in higher concentrations ALA exhibits the contrary effect. The latter results from a feedback control of porphyrin synthesis and from the fact that in the presence of ALA yeasts accumulate fluorescent metalloporphyrins which do not possess considerable photosensitizing activity. The supplementation of growing yeast culture with both ALA and 2,2'-dipyridyl, able to form the range of stable metal-ion complexes strongly sensitize cells to visible light. According to the spectrofluorometric analysis the amount of metalloporphyrins (except Mg-protoporphyrin) in the cells exposed to ALA and 2,2'-dipyridyl is decreased and coproporphyrin and protoporphyrin are accumulated. The certain type of endogenous porphyrins depends on ALA concentration: when exposed to ALA concentrations up to 0,1 mM yeasts accumulate both coproporphyrin and protoporphyrin IX, at higher concentrations mainly protoporphyrin IX. The accumulation of Mg-protoporphyrin IX does not inhibit the further synthesis of ALA-induced protoporphyrin that serves as the sensitizer in photodynamic damage to yeast cells.

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5-84

IS THE PARTITIONING EQUILIBRIUM OF POPC INFLUENCED BY BUFFER IONS ? A MONOLAYER STUDY. *S.E. Taylor*

Monolayers of the lipid Palmitoyl-oleoyl-phosphatidylcholine, POPC, may be considered as a basic model for the lipid matrix of natural membranes.

Recently POPC was found to undergo a surface pressure dependent partitioning equilibrium between the air/water interface and the aqueous subphase [1]. That study has been performed with McIlvaine buffer at pH 7 and an ionic strength of 60mM. The data presented here compare McIlvaine buffer with HEPES buffer both at pH 7 and an ionic strength of 170mM. There is no measurable difference between a 60mM or a 170mM McIlvaine buffer : at a surface pressure of 25mN/m the area per molecule is 78Å² ± 2Å² and the apparent partition coefficient $K_p = \Gamma/c_S$ = 16.5cm ± 1cm. For a similar HEPES buffer the situation is changed : at 25mN/m the area per molecule is 70Å² ± 2Å² and K_p = 13.9cm ± 1cm. The partition coefficients of both buffer systems indicate a slightly greater affinity of the lipid to the interface when a McIlvaine buffered subphase is used.

This might be explained with the formation of complexes between the buffer ions and the hydrophilic headgroups of the lipid. In the case of McIlvaine buffer a negatively charged complex between POPC and the citrate molecule can be assumed. Electrostatic repulsion of such an interfacial complex increases the area per molecule at a fixed lateral pressure. On the other hand the zwitterionic HEPES molecule cannot change the total charge of a POPC molecule and thus no increase in the area per molecule is observed.

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5-85

FRAP AT VARIABLE BEAM RADIUS : A POWERFUL TOOL FOR THE CHARACTERIZATION OF MEMBRANE DOMAINS.
A. Lopez, L. Salomé, J-F. Tocanne.

There is increasing experimental evidence that biological membranes are not organized at random but are structured in microdomains. We show that Fluorescence Recovery After Photobleaching (FRAP) experiments can be used to detect and characterize these membrane domains when they consist of closed lipid areas.

Numerical simulations of FRAP experiments at various observation radii Robs, have been carried out on a model membrane consisting of hexagonal closed areas of variable sizes r. From the data, relationships between the mobile fraction M, the diffusion coefficient D, the size of the membrane micro-domain r and the observation radius Robs, have been established. This numerical approach has been tested to be evaluated by performing FRAP experiments on a micro-compartmented experimental model, consisting of spherical supported vesicles constituted by a monolayer of silica beads supporting a phospholipid bilayer.

Consistingly, both the numerical and experimental approaches show that, if micron-sized closed lipid domains exist in a biological membrane, FRAP experiments performed at various Robs, enable to be recognized their existence and to be evaluated their size and diffusionnal characteristics.

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5-86

INCORPORATION OF AN AMPHIPATHIC PEPTIDE INTO PHOSPHOLIPID MONOLAYERS.
D.Trommeshauser, H.-J. Galla

The interaction of amphipathic peptides with membranes is important for many biological membrane related processes, for example the fusion process. Small amphipathic peptides are e.g. components of toxine mixtures like the bee venom mellitin or magainin, an anti-microbial frog peptide.

We investigated an amphipathic peptide from the carboxyterminal part of the envelope glycoprotein gp41 of HIV. This protein fragment interacts with membrane surfaces. The sequence of the peptide contains six positively charged arginine residues and several hydrophobic residues (RVIEVVQGACRAIRHIPRRIR).

To elucidate the peptide-lipid-interaction, we studied mixed peptide-lipid-monolayers by film balance measurements. The adsorption and insertion of the peptide into preformed lipid monolayers was studied with uncharged dipalmitoylphosphatidylcholine (DPPC) and negatively charged dipalmitoylphosphatidylglycerol (DPPG) as lipid matrix at different surface pressures.

In mixed peptide-lipid-monolayers the peptide is squeezed out of the air-water-interface at a surface pressure of about 30 mN/m. Below this surface pressure a significant insertion of the peptide added to the subphase of a preformed lipid monolayers containing negatively charged phospholipids can be observed. The peptide adsorbed to the monolayer by an electrostatical interaction between the positively charged arginine residues and the negatively charged phospholipids. After adsorption the peptide can incorporate into the monolayer. The same mechanism is valied for magainin and mellitin, so that the peptide we investigated, is a member of the group of small amphipathic peptides interacting with membranes and membrane surfaces.

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5-87

CHANGES IN ACTIVITY OF AN ENVELOPE ANION CHANNEL ASSOCIATED WITH CHLOROPLAST PROTEIN IMPORT.
P. J. W. van den Wijngaard and W. J. Vredenberg.

The chloroplast interior (stroma) is separated from the cytosol by the envelope membrane. Protein import across the chloroplast envelope has been shown to be associated with an electrical response of this barrier. Purpose of this research is to identify and characterise changes in ion channel activity during protein import.

Single channel recordings were obtained by using a standard patch clamp technique in the inside out patch configuration. Recordings were made in asymmetric (25/250 mM) KCl solution.

A 50 pS anion channel was found to be present in the chloroplast envelope. This channel could be blocked by the wild type precursor of ferrodoxin (wt-prefd) in the presence of 0.5 mM ATP. This blockade was concluded from a decrease in open time probability of the channel. The blockade by wt-prefd was ATP dependent; no effect was observed at ATP concentrations below 50 µM. In the presence of 100 µM ATP prefd blocked the channel to some extent. The transit sequence of ferrodoxin (trfd) alone also blocked the anion channel, but the decrease in open time probability was less than that caused by prefd. An import incompetent deletion mutant of prefd was unable to block the 50 pS anion channel.

It is concluded that 50 pS anion channel of the chloroplast envelope is involved in protein import into chloroplasts. The channel could be part of the protein import machinery. The ATP dependent blockade by wt-prefd suggest that an import competent precursor can bind to the channel during protein import. The smaller size of trfd as compared to prefd could be responsible for its lower blockade capacity.

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5-88

NEURON-SILICON JUNCTION WITH HIPPOCAMPAL NEURONS.
S.Vassanelli, P. Fromherz.

Neuron-silicon junctions were assembled hitherto from large neurons of the leech [1, 2]. It is an important step to apply the novel technique of extracellular recording to vertebrate neurons. We cultivate hippocampal neurons of the rat on small field-effect transistors. We study young cultures in order to avoid a perturbation by glia cells. The neurons are contacted electrically by a patch-electrode. Large voltage-pulses are applied as well as small AC-voltages. We are able to detect a response of the transistor in both cases. The voltage-pulse is recorded as a first derivative. The AC-signals are detected with a phase shift of 90°. In both cases the response is much weaker than in junctions with leech neurons. An evaluation shows that the seal resistance is similar but that the membrane capacitance in the junction is much lower. The result is compatible with the smaller size of the attachment site and the smaller specific capacitance of the membrane. Whether the contact is sufficient to record action potentials has to be seen.

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[2] P.Fromherz, A.Stett, Phys.Rev.Lett. 75 (1995) 1670.

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5-89

PUMP ACTIVITY OF THE MEMBRANE PROTEIN BACTERIORHODOPSIN AT REDUCED HUMIDITY

Stephan A. W. Verclas[1,2] and Norbert A. Dencher[2]

Bacteriorhodopsin (BR) is a transmembrane protein in the purple membrane (PM) of *Halobacterium salinarium*. When energized by light, BR generates a proton gradient across the cell membrane while undergoing a photocyle. Proton gradients play a fundamental role in energy transduction. The proton gradient is used by the H^+-ATP-synthases to produce ATP (adenosine-triphosphate), the universal energy storage in most metabolic processes of life. Water molecules are assumed to be an essential part of the proton pathway through the membrane. They can act as bridges where the distance between the protonable amino acid side chains is too long for an effective H^+-transfer.

To get information on the participation of water molecules in active proton translocation, we have measured the pump activity and the photocyle of BR in PM-films at reduced humidity (r.h.) between 100% and 15% r.h. by time-resolved absorption spectroscopy using optical H^+-indicators. We found that there is a minimal hydration neccessary for the pump activity: at 41% r.h. BR undergoes a photocycle, represented by the M-intermediate, however, the pump activity is strongly reduced compared with higher hydration. At 29% and 15% r.h. there is still a M-intermediate but no proton pumping. The kinetics of the proton pumping and the photocycle at different humidities are related.

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5-90

CONTROL BY PULSE DURATION OF THE LOSS OF CELL VIABILITY INDUCED BY ELECTROPULSATION: THE CASE OF CHINESE HAMSTER OVARY (CHO) CELLS.

M.C Vernhes, P.A Cabanes and J.Teissié.

Short lived electric pulses applied on cell suspension (electropulsation) induce a sharp increase in membrane permeability when strong field intensities are used. This electropermeabilization can be reversible but drastic conditions induce a loss in viability. It is well known that this loss can be dramatic when high intensities pulses are applied but the pulse duration is nevertheless controlling the process.

Square wave pulses were applied on CHO cells in suspension. Permeabilization was assayed by the penetration of the hydrophilic intercalant propidium iodide. Viability was evaluated in reference to unpulsed control cells. The influence of physical parameters (medium conductivity, post pulse temperature, osmotic pressure, vectoriality of the field) was evaluated. A systematic investigation of the effect of the pulse duration and number at a given field strength was run. For a given cumulated pulse duration, one single pulse was less damaging than a train of very short pulses at an 1Hz frequency.

As a conclusion, the loss in viability is not related to the energy delivered to the system during the pulsation. An accumulation of small stresses is very effective to induce the loss in cell viability. Control experiments showed that these differences in the damaging effects of the field are not related to the temperature increase, to the level of permeabilization or to the oxidative stress. (The work was supported by a grant of the Service des études médicales of E.D.F.)

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5-91

A NEW APPROACH FOR CELLULAR DELIVERY OF POLYNUCLEOTIDES USING SYNTHETIC PEPTIDES.

P. Vidal, M. Morris, L. Chaloin, J. Méry, F.Heitz and G. Divita.

The major difficulty for the delivery of nucleic acids into intracellular domains still remains in the crossing of the cell membrane. We describe here a new potent strategy for nucleic acids delivery into cells using a short synthetic amphipathic peptide derived from both a fusion protein and a nuclear localization sequence. The role of each these sequences is respectively to anchor the peptide to the lipidic membrane and to promote nuclear addressing of the peptides. Using a fluorescent probe covalently attached to the peptide through a C-terminal cysteamide function it is shown that these peptides can be internalized by cells with a predominant nuclear localization. The formation of complexes between the peptide and nucleic acids was quantified using both the intrinsic fluorescence of the single Trp of the peptide and fluorescently labelled mRNA or oligonucleotides. The vector peptide exhibits a relatively high affinity for both oligonucleotide and mRNA (in a 100 nM range) and the complex thus formed contains a ratio of one peptide per 4-5 base units depending on the nature of the nucleic acid. This result suggests that, beside the peptide - polynucleotide interactions which are mainly governed by electrostatic interactions, peptide - peptide interactions, which do not occur in the absence of polynucleotide, may also take place leading to a large particle which shows mainly the peptide properties. In the presence of the vector peptide both fluorescently labelled mRNA and oligonucleotides are efficiently delivered into mammalian cells in less than 1 hour which is considerably lower than that required when using lipofectamine (≈ 4 hours).

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5-92

MEMBRANE EFFECTS OF PORPHYRINE-TYPE PHOTOSENSITIZERS

I. Voszka, G. Csik, E. Balog, P. Maillard, M. Momenteau

The effect of several glycosilated porphyrine derivatives, possibly applicable in photodynamic therapy of tumors was examined on biological and model membranes. Red blood cells' hematological parameters (red blood cell concentration, hematocrit value, mean cell volume, etc.) were measured (a) after dark incubation with porphyrines and (b) after consequent irradiation. We found lack of dark effect. Using high intensity white light irradiation we found hemolysis and increase of mean cell volume. The strength of the effect was dependent on the structure of side chains of porphyrine derivatives. Liposomes were made from dimiristoyl-phosphatidylcholine by sonication. The interaction of photosensitizers with membrane was followed measuring the fluorescence anisotropy of either anilino-naphthalenesulfonic acid (ANS) or diphenyl-hexatriene (DPH), giving information about the lipid headgroup and carbohydrate chain region respectively. Measuring the temperature dependence of the fluorescence anisotropy we found, that tetraglycosilated porphyrins with either globular or planar structure are not able to penetrate the lipid bilayer. At least one apolar ring system among the substituents is required to get localisation in the apolar region of the lipid bilayer. Measuring the lipid phase transition parameters with microcalorimeter similar results were obtained. Comparing the results of measurements on blood cells and liposomes we found good coincidence.

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5-93

FRAP OF A FLUORESCENT TRANSFERRIN ENDOCYTOSED IN LIVING CELLS. F. Azizi and Ph. Wahl

The FRAP of Transferrin labelled with Lissamine Rhodamine Sulfochloride (Tf-LRSC) and internalized in the recycling compartment of Transferrin endocytosis was measured in living A431 cells. In cells treated with nocodazole or metabolic inhibitors, there remained a residual FRAP which was ascribed to the spontaneous reactivation of the bleached molecules. We showed that the fractional FRAP characterizing the intracellular transport of Tf-LRSC was obtained by subtracting the fractional FRAP of the nocodazole treated cells from the fractionnal FRAP of the non-treated cells. This active transport was interpreted by a mechanism involving carrier vesicles budding from stationary vacuoles, saltating along microtubules and fusing with other stationary vacuoles, according to previous video-microscopy observations. Assuming that the rate of transport was controlled by the fission-fusion between vesicles and vacuoles, we showed that the theoritical FRAP was an exponential function of time which we fitted to our experimental data. The number of vesicles fusing with a unit of vacuole surface was calculated to be 0.15 μ^{-2} sec^{-1}. The rate of the fusion was divided by two in cells treated with AlF_4^-, and increased to 20% in cells treated with Brefeldin A. The fusion process appeared to be homotypic and regulated by an heterotrimeric G protein.

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5-94

MODIFICATION OF BILAYER PHOSHOLIPID MEMBRANES BY TETRACOSAPRENOL (C_{120}). Krystyna Walińska, Teresa Janas and Tadeusz Janas.

In the present study, we investigated macrovesicular bilayer membranes in the form of hemispheres, made from dioleoylphosphatidylcholine (DOPC) or its mixture with long-chain polyprenol - tetracosaprenol (C_{120}) and the influence of this polyprenol on capacitance, permeability and stability of bilayer lipid membranes (BLM). The behaviour of C_{120}/DOPC membranes has been studied using voltametric techniques. The current-voltage characteristics, steady-state diffusion potentials, membrane conductance-temperature relationships, electric capacitance and breakdown voltage have been studied. The ionic transference numbers for Na^+ and Cl^- ions, the activation energy for ion migration, membrane hydrophobic thickness and the membrane Young's moduls have been determined. Tetracosaprenol (C_{120}) increases membrane normalized conductance, membrane ionic permeability, membrane elastic deformability and the membrane hydrophobic thickness, decreases the activation energy for ion transport, membrane specific capacitance and membrane electromechanical stability, and does not change membrane selectivity. The results show that the transmembrane electrical potential does play an important role in the dynamics and conformation of polyisoprenols in membranes. Long-chain polyprenols can modify bilayer lipid membranes by the formation of fluid microdomains.

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5-95

INFLUENCE OF DIBUCAINE ON THE SURFACE POTENTIAL AND SURFACE PRESSURE OF LIPID MONOLAYERS

Ingrid Weis, Gerhard Wackerbauer and Mathias Winterhalter

We investigated the influence of Dibucaine, a local anesthetic, on the surface potential and surface pressure of lipid monolayers. In a first series of measurements increasing amounts of Dibucaine were injected into the aqueous subphase. Subsequently the surface pressure as well as the surface potential was recorded. In a second series of measurements lipid monolayers were spread in various concentrations on the air-water interface and after equilibration Dibucaine was injected into the subphase. The amount of Dibucaine in the air-water interface or lipid-water interface is well described by a partition coefficient and our data on the surface pressure agrees with those previously published. Moreover, we show that recording the surface potential allows conclusion below mM concentration at which no change in the surface pressure was visible. We show that even a µM concentration of Dibucaine causes a significant increase of the surface potential. This finding suggests that electric field effects eventually play a more prominent role in the local anesthetic action than previously expected. One possible action of the increase in electrostatic potential could be mechanically. Assuming that this potential drops in a narrow part of the bilayer this might cause large local mechanical stress inside this plane which might trigger conformational changes of proteins. On the other hand small variation in the electric field distribution might influence the partitioning of other substances like Ca^{2+}.

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MEMBRANE INTERACTIONS OF THE BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN BPI. A. Wiese, K. Brandenburg, E. Th. Rietschel and U. Seydel.

We have investigated the mechanisms of interaction of the recombinant N-terminal portion of BPI, $rBPI_{21}$, (XOMA Corporation, Berkeley, CA) with the outer membrane of Gram-negative bacteria. Experimentally, the action of $rBPI_{21}$ on various planar asymmetric and symmetric bilayer membranes including a reconstitution system of the lipid matrix of the outer membrane and on lipid monolayers at the air/water interface was analyzed. We have further studied the interaction of $rBPI_{21}$ with Re-mutant lipopolysaccharide (LPS) aggregates by applying infrared and resonance energy transfer spectroscopy. We found that the addition of $rBPI_{21}$ to the LPS-side of asymmetric LPS/phospholipid membranes as well as to black lipid membranes made from dioleoylphosphatidylglycerol (DOPG) led to membrane rupture. The innermembrane potential difference derived from current/voltage-curves of membranes doped with the K^+-carrier nonactin gave a slight increase from 0 mV to 5 mV for symmetric DOPG-membranes, but changed for asymmetric LPS/PL-membranes from -36 mV to +8 mV following the addition of $rBPI_{21}$. In both cases, the addition of $rBPI_{21}$ led to an increase in membrane current. Furthermore, $rBPI_{21}$ caused the rigidification of the acyl chains of LPS- and phosphatidylglycerol (PG) in the gel as well as in the fluid phase and a drastic immobilization of their phosphate groups. From monolayer experiments, the molecular area of a single $rBPI_{21}$-molecule was estimated to be about 12 nm^2. At lateral pressures of $\leq$ 25 mNm^{-1}, $rBPI_{21}$ incorporated into LPS- and PG- but not into neutral lecithin monolayers. Also, $rBPI_{21}$ could be shown to incorporate into liposomes containing negatively charged phospholipids. In all systems, high concentrations of Mg^{2+}-ions had a protective effect. On the basis of these results, we propose that membrane rupture, increase of membrane current, and change of transmembrane potential as consequence of $rBPI_{21}$-action may contribute to bacterial dysfunction.

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ROLE OF THE SURFACTANT PROTEIN C IN THE STABILIZATION OF LOW SURFACE TENSIONS AT THE ALVEOLAR AIR/WATER INTERFACE. A. Wintergalen, M. Amrein, A. von Nahmen, S. Krol, H.-J. Galla and M. Sieber.

Purpose: Pulmonary surfactant - a complex mixture of lipids and proteins - reduces the surface tension at the air/water (a/w) interface dynamically during the breathing cycle. This behaviour can be explained by the ability of the surfactant protein C (SP-C) to form lipid/protein multilayers at the interface. We investigated artificial films formed at the a/w interface by film balance measurements, scanning force microscopy (SFM), secondary ion mass spectrometry (SIMS) and fluorescence light microscopy (FLM). Here, the results of topographical investigations by SFM are discussed.

Methods: The surface properties of surfactant analogues films consisting of phospholipids (DPPC/DPPG in the ratio 4:1) and SP-C were studied using film balance techniques. In order to investigate the topography by SFM, the films were transferred from the air/water interface to a solid support by the Langmuir-Blodgett (LB) and the Langmuir-Schäfer technique. Dependent on the transfer technique, the structure of the films was visualized in gas or in liquid.

Results: The most striking characteristic of the isotherms recorded on compression of the film is a plateau region with extremely high compressibility at high surface pressures ($\Pi \cong 50$ mN/m) and low molecular areas. No loss of material was observed in succesive compression/expansion cycles. The SFM images of films transferred in the plateau region show a phase separation. One of the two phases consists of multilayer stacks.

Conclusions: In the plateau region of the isotherm a reversible collapse of the surfactant analogues film occurs on compression. During the collapse process, surfactant material enriched in protein (shown by FLM) accumulates in form of multilayer stacks. From a physiological point of view, these stacks act as a surfactant reservoir which forms on exhalation and respreads on inhalation.

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5-98

PORE-FORMING ACTIVITY OF SPIRALIN, A SPIROPLASMA MEMBRANE ANCHORED LIPOPROTEIN. L. Béven,* H. Duclohier,† C. Brenner,* M. Le Hénaff * and H. Wróblewski *.

Spiroplasmas are helical, wall-less bacteria related to the mycoplasmas and containing an intracytoplasmic bundle of protein fibrils extending axially the entire length of the cell. The outer face of their plasma membrane is crowded by spiralin molecules anchored to the lipid bilayer by three fatty acyl chains attached to an *N*-terminal *S*-(2,3-dihydroxypropyl)cystein. Spiralin (219 amino acid residues) and the fibril protein (515 residues) are unrelated to any known protein. We have previously shown that synthetic copies of a 20-residue amphipathic α helix of spiralin formed concentration- and voltage-dependent pores in planar bilayers, with a unitary conductance of 0.13 nS in 1 M KCl. We show here that purified spiralin is also capable of forming in the same conditions concentration- and voltage-dependent pores, but with a unitary conductance of 6 nS. Since the presence of such pores in the plasma membrane would be lethal for the spiroplasma cell as it would dissipate the transmembrane electrochemical gradient, we infer that spiralin molecules form a network of pores *on* the surface of the cell and are bound to the plasma membrane by their lipoyl moeity. This "velcro-like"model which suggests that spiralin is the component of novel type of bacterial S-layer, is consistent with a theoretical analysis of the amino acid sequence of the protein and is supported by experimental data on spiralin topology and spiroplasma membrane energetics.

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5-99

EFFECTS OF INTRACELLULAR ELASTIC FIBRES ON THE SHAPES OF PHOSPHOLIPID VESICLES AND RED BLOOD CELLS. B. Božič, D. Grabec, S. Svetina and B. Žekš.

The equilibrium shapes of phospholipid vesicles and red blood cells are determined by the requirement of the minimal membrane elastic energy, that consists of the local membrane bending energy and of the nonlocal bending energy characteristic for bilayer and multilayer closed membranes. The equilibrium shapes are usually axially symmetric and can be either prolate or oblate.

When the elastic fibres are in the cells or in the vesicles, the minimization procedure must include also the bending and the stretching energy of the fibres, that therefore produce some forces on the membrane and influence the cell shape. The fibres, that are rigid enough, prefer the prolate shapes. They tend to elongate the cells by axial forces and can produce the tethers in both directions of the axis. In some cases the tethers can be asymmetric breaking the mirror plane symmetry of the cell. When the axial forces exceed a critical value, the fibres bend and break also the axial symmetry of the cell. Less rigid or longer fibres tend to arrange equatorially, conserving the axial and the mirror plane symmetry of the shape and tending to increase its equatorial radius.

The obtained theoretical results can be usefull for better understanding of the properties of the sickle cell erythrocytes and of the role of the marginal band in nucleated erythrocytes.

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6-1

ROLE OF ELECTROSTATICS AND PROTEIN DYNAMICS IN ELECTRON TRANSFER IN PHOTOSYNTHETIC REACTION CENTER. E. Frolov, V. Goldanskii and F. Parak.

The interheme electrostatic interaction and protein dynamics can explain some peculiarities of the electron transfer (ET) from the cytochrome subunit to the photooxidized bacteriochlorophyll dimer (P) in the reaction center of Rps. viridis, as: *i.* low temperature restriction of ET; *ii.* its restoration and *iii.* acceleration of ET.

The acceleration of electron tunneling under reduction of several hems in cytochrome subunit is caused by the shifting of the electron level of heme-donor due to interheme electrostatic interaction which leads to decreasing of the tunneling barrier height. The electrostatic interaction energies calculated for neighbouring hemes, 7.0 Å apart and for two high potential hemes, 21.5 Å apart are found to be 0.11 eV and 0.04 eV respectively. The reorganization energy of the transition of about 0.29 eV is calculated according to Marcus theory of electron tunneling. An empirical relation for the rate of ET is given.

The low temperature restriction of ET is caused by an energetic ban which originates from a shifting of the energy levels of P due to the freezing of protein dynamics. As a result the out-of-equilibrium state of P is initiated under its photoinduction at low temperature. The electronic level of such state differs from its equilibrium state on the value of reorganization energy of P formation equal to 0.13 eV which is higher than the free energy of reaction equal to 0.12 eV at room temperature. It leads to the energetic ban which blocs tunneling. Under reduction of the neighboring heme(s) the energetic ban is vanished due to electrostatic shift of electronic level of heme-donor and ET is reinstated. The freezing of the protein dynamics is displayed by the Mössbauer effect and correlates with the efficiency of the ET.

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6-2

STRUCTURE OF PHOTOSYNTHETIC MEMBRANE PROTEIN COMPLEXES DETERMINED BY ELECTRON MICROSCOPY

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Electron crystallography of two-dimensional crystals is used increasingly to determine the structure of membrane proteins which are proving elusive for three-dimensional crystallisation and x-ray crystallography.

This method is being used to determine structure of two photosynthetic membrane proteins from green plant chloroplasts: the light-harvesting chlorophyll *a/b*-protein complex (LHC-II) and the photosystem II reaction centre core complex (PSII RC). The structure of LHC-II has been determined at 3.4 Å resolution. LHC-II binds roughly half of all the chlorophylls involved in plant photosynthesis and uses them to collect solar energy which is passed on to the photosystem II reaction centre. The atomic model of the complex shows the structure of three membrane-spanning helices, a short amphipathic helix at the membrane surface, and the non-covalently bound chlorophylls and carotenoids. The two central helices are connected through symmetrical ion pairs which also function as chlorophyll ligands. In the centre of the monomer, 7 of the 12 bound chlorophylls, assigned to Chl *a*, are in van-der-Waals contact with two symmetrically placed carotenoids.

The structure of the PSII RC has been determined in projection at 8 Å resolution. The projection map indicates the position of the two proteins, D1 and D2, which are thought to bind the chlorophylls involved in electron transport across the membrane.

6-3

J. L. Martin.

No abstract available.

6-4

THE INTERACTION BETWEEN CYTOCHROME C_2 AND THE PHOTOSYNTHETIC BACTERIAL REACTION CENTER: TESTING INTERPROTEIN ELECTRON TRANSFER MODELS. G. Venturoli, F. Drepper, P. Mathis, J. Allen, X. Lin

The reaction between cytochrome (cyt) c_2 and the photoxidized primary donor P of the reaction center (RC) has been extensively characterized and provides a model to study electron transfer between a small soluble protein and a multiprotein complex. The kinetics of the reaction are well described by a scheme which includes a collisional process and a first order electron donation within a cyt c_2-RC complex. To test theoretical models of electron transfer, we studied the rate of the intracomplex process as a function of temperature (240K<T<300K) and of the driving force $-\Delta G°$, by using a series of specifically mutated RC from *Rhodobacter sphaeroides* characterized by a range of altered redox potentials of P/P^+ from +410 mV to +765 mV (compared to +505 mV for wild type). The kinetics of the reaction have been studied by flash absorption spectroscopy, monitoring the re-reduction of P^+ induced by a laser pulse. Rate constants (k) of the intracomplex reaction were obtained by multiexponential deconvolution of the kinetics. At all temperatures the change in $\Delta G°$ has a dramatic effect on k, which is found to increase by more than two orders of magnitude upon varying $-\Delta G°$ from 65 meV to 420 meV. Global analysis of T and $\Delta G°$ dependence in terms of the semiclassical Marcus equation yields a reorganization energy $\lambda=0.96\pm0.07$ eV and a set of electronic matrix elements, specific for each mutant, ranging from $1.2\cdot10^{-4}$ eV to $2.5\cdot10^{-4}$ eV. A comparable quality of the global fit can be obtained by using the Jortner equation only at low or intermediate frequencies of the vibrational modes coupled to the reaction (lower than 400 cm^{-1}). At this upper limit a quantum-mechanical description of the data requires moderately higher values of the reorganization energy and of the electronic matrix elements.

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6-5

SALT EFFECT OF COOPERATIVITY IN THE BACTERIORHODOPSIN PHOTOCYCLE. Zs. Ablonczy, E. Papp

The mutual effect of salt and actinic light on the M state kinetics of the bacteriorhodopsin photocycle was studied at different temperatures and bulk pH. High time resolution M state absorption-change measurements and local data analysis of the M state kinetics were applied for the studies. It was found that the cooperativity phenomenon depends strongly on the concentration of salt in the suspension.

At high concentrations the kinetics showed regular Arrhenius type behaviour while at low concentrations irregularities were found which correlated with the effect of salt on the dependence of the relative amplitudes of the kinetic components on the cycling fraction of bacteriorhodopsin molecules. Opposing the case of high salt at low concentrations not only the relative amplitudes but also the rate constants of the components changed with the exciting light energy. It can be shown that the dependence of the cycling fraction on actinic light energy is due to the back reaction of the K intermediate to ground state bacteriorhodopsin and therefore the cooperative effect is caused only by the average number of cycling bacteriorhodopsin molecules. The results indicate a general cooperative interaction already before and during the formation of the M state and a branching photocycle.

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6-6

SEARCH FOR A QUINONE TYPE SECONDARY ELECTRON ACCEPTOR IN HELIOBACTERIA. K. Brettel, W. Leibl and U. Liebl.

The photosynthetic reaction centre of heliobacteria bears many similarities to the reaction centre of photosystem I (PS I). There is good evidence that heliobacteria contain a BChl *g* dimer (P798) as primary electron donor (analogue to the Chl *a* dimer called P700 in PS I), a 8'-OH-Chl *a* as primary electron acceptor A_0 (Chl *a* in PS I) and three [4Fe-4S] clusters F_X, F_A and F_B, similar to the terminal acceptors in PS I. In PS I, a phylloquinone (A_1) serves as intermediate between A_0 and F_X; A_0^- reduces A_1 within about 30 ps, followed by electron transfer from A_1^- to F_X within 20 to 200 ns. The photosynthetic membrane of heliobacteria does not contain phylloquinone, but several menaquinones. We tried to establish whether a menaquinone is involved in physiological electron transfer from A_0^- to F_X in heliobacteria.

Membrane fragments from *Heliobacillus mobilis* were studied at room temperature by flash absorption spectroscopy with a time resolution of about 2 ns. Electron transfer from the semiquinone anion of a menaquinone to F_X is expected to give rise to a broad bleaching at around 400 nm. However, we were unable to detect any significant bleaching phase in the time window from 2 ns to 5 µs in the wavelength range between 360 and 450 nm. These data indicate that menaquinone is most likely not involved in electron transfer from A_0^- to F_X in *Heliobacillus mobilis*. Alternatively, the reoxidation of a hypothetical reduced menaquinone by F_X would have to be either much faster (< 2 ns) or much slower (> 5 µs) than that of A_1^- in PS I.

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6-7

MITOCHONDRIAL UNCOUPLING PROTEINS Fleury C., Levi-Meyrueis C., Ricquier D. and Bouillaud F.

The brown adipose Tissue is a thermogenic organ of mammals. In brown adipocytes there is expression of a mitochondrial uncoupling protein (UCP). UCP is a member of the family of mitochondrial anion carriers. UCP creates a pathway that allows protons to reenter the matrix without energy conservation. UCP activity is inhibited by nucleotides and is activated by free fatty acids. Recombinant expression of the UCP in Saccharomyces cerevisiae has been obtained. The use of potential sensitive probes in flow cytometry allows the study of mitochondrial activity in living yeast cells. UCP coding sequence was mutagenized randomly by PCR. Mutations that increase or abolish UCP activity *in vivo* were defined by cell sorting of yeast cells.

Existence of a mitochondrial carrier expressed in various tissue and 60% identical to the UCP has been suggested by systematic sequencing of cDNAs. We have cloned the full length cDNA of this protein in a library of cDNAs from mouse muscle (gb U69135). The term UCP2 was proposed for this protein (Fleury et al Nature Genetics March 1997), accordingly brown fat UCP is now called UCP1. Overexpression in S. cerevisiae of UCP2 indicates that this protein lowers the mitochondrial membrane potential, and decreases growth efficiency like UCP1 does. These effects are not seen when inactive mutants of the UCP1 are overexpressed and this rules out a mere effect of overexpression of a foreign protein in mitochondria.

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6-8

ELECTROGENIC EVENTS ASSOCIATED WITH PEROXY - TO - FERRYL - OXO STATE TRANSITION IN CYTOCHROME C OXIDASE. S.A. Siletsky, A.D. Kaulen and A.A.Konstantinov.

The 3-th and 4-th electrons that reduce the "peroxy" complex (P) to the Ferryl-Oxo state (F) and then F to the Oxidized (Ox) state, respectively, are supposed to drive electrogenic proton pumping in cytochrome c oxidase (COX). Earlier we have characterized 3 electrogenic phases associated with the flash-induced reduction of F to Ox with the aid of time-resolved electrometric method of monitoring membrane potential. Flash-induced reduction of P (formed by CO bubbling method) to F gives rise to generation of membrane potential that includes 3 major electrogenic phases with characteristic times of about 50 mks, 0.7-0.9 ms and 2.5-3.5 ms which is close to the response observed for the F-to-Ox transition (40 mks, 1.2 ms and 4 ms, respectively, at pH 8). The microsecond phase is due to the vectorial electron transfer from CuA to haem a while the two millisecond phases reflect vectorial proton translocation linked to reduction of haem a3-bound oxygen intermediate. This reduction of haem a3 in P-to-F transition is about several fold slower than conversion of P to F during the oxidation of the fully reduced enzyme by O2 in the flow-flash experiments but agrees with the results of Nilsson for RuBpy-mediated flash-induced reduction of P generated by CO bubbling method. The discrepancy may emphasize the role of redox state of CuB in P; in the flow-flash O2 oxidation measurements [4] CuB in the P state is most probably cuprous, whereas in experiments with photoreduction of P generated by CO bubbling, CuB is most likely to be in the oxidized state.

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6-9

A STUDY OF THE ADAPTATION OF THE CYANOBACTERIUM *SYNECHOCOCCUS* PCC 7942 CYTOPLASMIC MEMBRANE TO DIFFERENT NITROGEN SOURCES BY LASER DOPPLER ELECTROPHORESIS

M. Zinovieva, C. Fresneau and B. Arrio

Several years ago, a NO_3^- dependent 47 kDa polypeptide in *Synechococcus* cytoplasmic membrane was described. Recenly, we found that another 126 kDa polypeptide existed in the cytoplasmic membrane of the *Synechococcus* PCC 7942 cells grown in the presence of NO_3^-. The presence of NH_4^+ inhibited the expression of this protein. The amount of 126 kDa polypeptide was inversely related to the NO_3^- concentration. Transfer of NH_4^+ grown cells to a NO_3^- containing medium restores the expression of both polypeptides. Similar effects were observed for $NaNO_2$. The expression of these proteins was time dependent. These changes of the protein composition exerted an influence on the number of electrostatic charges at the membrane surface resulting in electrophoretic mobility variations measured by laser Doppler electrophoresis. The study of the cytoplasmic membrane electrophoretic mobility and charge density in the absence and in the presence of various salts showed a specific interaction of NO_3^-. This interaction may be a binding or a structural arrangement of the membrane surface. It was more pronounced in the membrane from the cells grown at low NO_3^-. The data obtained suggest that composition of surrounding medium may regulate the nitrate affinity of transport system(s).

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6-10

CONTRIBUTION OF SUBUNIT PsaE TO THE BINDING OF FERREDOXIN TO PHOTOSYSTEM I. P. Barth, B. Lagoutte, H. Bottin, P.M.U. Sétif

The process of ferredoxin reduction by photosystem I has been investigated in detail by flash-absorption spectroscopy in a *psaE* deleted mutant from *Synechocystis* sp. PCC 6803. In this mutant, the dissociation constant for ferredoxin binding is increased 100-fold compared to the wild type whereas the second-order rate constant is only reduced twice. This suggests that the absence of PsaE results mostly in a large increase of the dissociation rate. However, the kinetics of electron transfer within the PSI/ferredoxin complex are very similar in the deleted mutant and in the wild type, which indicates that the geometry of the PSI/ferredoxin complex is not affected by the absence of subunit PsaE. Site mutated *psaE* genes were used to transform the deleted strain in order to obtain a new series of mutants. Among them, two single-site mutants were found to be strongly modified in their affinity for ferredoxin. Photosystem I in which arginine 39 of PsaE has been replaced by glutamine behaves almost identically to the PsaE deleted mutant whereas the addition of an extra arginine in the place of tryptophan 17 induces a 20-fold affinity increase for ferredoxin (dissociation constant of 0.025 μM). These results indicate that PsaE, probably via a single arginine residue at position 39, is essential for tuning the ferredoxin dissociation constant in the micromolar range.

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6-11

INTERACTION OF THE REDUCED CYTOCHROME BD FROM E.COLI WITH CYANIDE. V.B. Borisov, J.P. Osborne, S.A. Siletsky, R.B. Gennis and A.A. Konstantinov.

Cytochrome bd (BDOX) is one of the two membrane quinol oxidases of E.coli respiratory chain. According to Rich and collaborators [1], BDOX differs from the oxidases of the heme/copper superfamily in that it does not bind KCN in the reduced state. In contrast, evidence for KCN reaction with the fully reduced BDOX (rBDOX) was reported in [2]. We show that KCN reacts with rBDOX under anaerobic conditions bringing about absorption spectral changes indicative of ligand binding to heme d. These changes titrate with apparent Kd of 50 - 100 mM and are reversed upon dilution of the ligand-pretreated enzyme. The kinetics of the spectral changes is biphasic. There are an unresolved fast phase (~20%) and a major slow transition (~80%). The contribution ratio of the two phases does not change significantly with [KCN]. The rate of the slow phase depends linearly on [KCN] in the range 5 - 100 mM giving kon = 0.05 - 0.1 M-1s-1 and koff = 0.003 s-1. CO added to the cyanide complex apparently extrudes KCN and CO adduct of rBDOX is formed with 80-90% yield. However, much more CO is required to saturate formation of the CO complex in the presence of 100 mM KCN as compared with CO binding with the free rBDOX. The two ligands compete with each other for binding with heme d. Cyanide compound of rBDOX does not show photodissociation in the microsecond scale.

[1]. Mitchell et al. (1995) Biochemistry 34, 7576-7585.
[2]. Sun et al. (1996) Biochemistry 35, 2403-2412.

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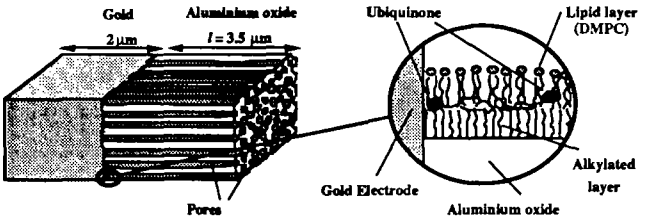
6-12

AN ELECTROCHEMICAL APPROACH OF THE LATERAL MOBILITY OF UBIQUINONES AND PLASTOQUINONES INCPORPORATED IN SUPPORTED PHOSPHOLIPID LAYERS. D. Marchal, W. Boireau, J.M. Laval, J. Moiroux, and C. Bourdillon.

Physiological mole fractions of long isoprenic chain ubiquinone (UQ_{10}) and plastoquinone (PQ_9) were incorporated inside a supported bilayer by the method of vesicle fusion. The bilayer was laterally in contact with a built-in gold electrode at which direct electron transfers between the redox heads of the quinones molecules and the electrode can proceed (figure).



The mass balances of quinone and lipid in the structure were determined by radiolabeling and spectrophotometry. The mole fraction of quinone was between 0.5 and 3 mol%, remaining unchanged when going from the vesicles to the supported layers. The lipid molecules and the quinones pool were both laterally mobile. Cyclic voltammetry was used to investigate the redox properties of UQ_{10} and PQ_9 over a wide pH range. Within the experimental uncertainty, the standard potentials and the pK_a's of the pertinent redox forms of UQ_{10} and PQ_9 were found essentially identical. This differs slightly from the literature in which the constants were deduced with quinones in mixed solvents. The lateral mobility of the unlabeled UQ_n was studied by chronocoulometry. The long rage diffusion coefficients of UQ_n were found in the range 2 to 5 10^{-8} cm^2 s^{-1}, directly linked to n, the number of isoprenic units. A simple model of quinone diffusion in the midle plane of the bilayer explains the influence of the chain lengths.

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6-13

CHARACTERISATION OF THE STRUCTURAL AND FUNCTIONAL ORGANISATION OF THE CYTOCHROME b_6f COMPLEX. C. Breyton, J.-L. Popot

A protocol of purification of the cytochrome b_6f complex from the thylakoid membranes of *Chlamydomonas reinhardtii* has been developed, which gives a highly pure and active complex. Besides the four large subunits, we detect three mini-proteins of around 4 kDa. A molecule of chlorophylle *a* has been shown to be a genuine component of the purified complex; its role is still a mystery. The molecular weight of the purified complex has been determined by combining velocity sedimentation and molecular sieving analyses and determination of its lipid and detergent content. The purified complex is an active dimer. Upon delipidation, the dimer converts irreversibly into an inactive, monomeric form that has lost two of the subunits and the molecule of chlorophyll. The purified preparation appears suitable for crytallisation : 2D crystals have been grown following reconstitution of the b_6f dimer into lipid vesicles. After negative staining, they diffract to at least 10 Å resolution. A projection map shows that the b_6f complex crytallises as a dimer.

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Purification and Characterisation of the cytochrome b_6 f complex from *Chlamydomonas reinhardtii* Y. Pierre, C. Breyton, D. Kramer, and J.-L. Popot *J. Biol. Chem.* **270**, 29342-29349, 1995
Projection map of cytochrome b_6f complex at 8 Å resolution. G. Mosser, C. Breyton, A. Olofsson, J.-L. Popot, and J.-L. Rigaud, *submitted*
Dimer to monomer conversion of the b_6f complex : causes and consequence. C. Breyton, C. Tribet, J. Olive, J.-P. Dubacq and J.-L. Popot, *submitted*
On the presence, role and evolutionary origin of a molecule of chlorophyll a in the cytochrome b_6f complex. Y. Pierre, C. Breyton, B. Robert, C. Vernotte, and J.-L. Popot, *submitted*

6-14

IMPLICATIONS OF PROTON RETARDED TRANSFER FROM PURPLE MEMBRANE INTO THE AQUEOUS BULK PHASE FOR ENERGY-COUPLING THEORIES. K. Bryl and K. Yoshihara

According to the chemiosmotic hypothesis, an intermediate state in ATP synthesis *via* F_0F_1ATPase is a proton gradient. The protons are either delocalized in the bulk medium or localized at the site of the enzyme complex within the membrane. *Halobacterium salinarium* cells have the advantages for testing energy coupling concepts that bacteriorhodopsin (bR) is the best established example of an electrogenic proton pump and that it occurs in distinct crystalline patches (purple membranes) in the coupling membrane, hindering any direct interaction of the proton pump with the ATPase. The aim of study was to clarify whether protons can diffuse between a source (bacteriorhodopsin) and a sink (H^+-ATP-synthase) without dissipation losses into the aqueous bulk and whether ATP can be produced due to such a coupling. The experiments with liposomes containing H^+-ATP-synthase, bR or modified bR (wild-type and mutant D96N regenerated with 14-Fluororetinal) were performed. The H^+ ejection kinetics and H^+ transfer were monitored with optical pH indicator covalently linked to the surface of bR and optical pH indicators residing in the aqueous bulk phase. The light-driven ATP synthesis was monitored with luciferin-luciferase test. The results demonstrate that modified bacteriorhodopsins can undergo light-induced cyclic reactions without direct releasing protons to the aqueous bulk. Protons after reaching the surface of membrane, diffuse along the membrane and only part of them is transferred into the aqueous bulk. The level of ATP production was very similar in all investigated systems. This result suggests that some fraction of the bR molecules may by coupled with ATPase *via* diffused protons.

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6-15

ELECTROGENIC ACTIVITY OF PATCH-CLAMPED CHLOROPLAST IN RELATION TO LIGHT-INDUCED STRUCTURAL CHANGES OF THYLAKOID SYSTEM. A.A. Bulychev,[1,2] J.H.A. Dassen[2] W.J. Vredenberg[2], V.K. Opanasenko[3] and G.A. Semenova[4].

The photocurrent in Peperomia metallica chloroplasts is modified by replacement of external KCl (50 mM) with NH_4Cl. At zero holding potential, an initial rise of inward current was followed by a slow (~1s) rise to a level nearly twice the initial peak, whereas in the presence of KCl, the photocurrent declined during illumination. Upon intermittent lighting, the photoresponse decreased with a pulse number under control conditions but increased in NH_4-treated samples. The current induced by 6-us flach in the presence of NH_4^+ was 50-100 % higher in preilluminated than in dark-adapted plastids. Hence, the apparent photoactivation of electrogenic activity was not due to acceleration of electron turnovers. Either (1) latent RCs turn active during preillumination or (2) the pipette becomes more effective sink for current, owing to NH_4^+-dependent swelling of lumen and the respective reduction in the longitudinal lumenal resistance. In support of this view, the flash-induced response in NH_4^+ treated plastids had shorter relaxation time in preilluminated than in dark-adapted plastids (35.1 and 72.3 ms, respectively). Thus, the light-induced structural changes, confirmed by EM micrographs of the thylakoid system, strongly affect the photocurrents probed by the patch-clamp technique. The dynamic properties of the thylakoid are apparently of importance for spacial distribution of generated currents and consequently for the bioenergetic performance of the pastid.

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6-16

PURIFICATION OF SERCA-1a EXPRESSED IN YEAST. S. Chenevois, P. Falson, F. Corre, L. Bouneau, M. le Maire and F. Guillain.

Serca1a, the Ca^{2+}-ATPase of sarcoplasmic reticulum, was expressed in *Saccharomyces cerevisiae* (Centeno *et al.*, FEBS Letters 354, 117-122), with an additional C- or N-terminal tag of 6xHis. The Ca^{2+}-ATPase represents around 0.5% of total membrane proteins and can be purified combining affinity chromatography and ion-exchange chromatography. Ca^{2+}-ATPase was solubilized from yeast membranes using octa-ethyleneglycol mono n-dodecyl ether ($C_{12}E_8$) and selectively adsorbed on a Ni^{2+}-nitriloaceticacid (NTA) column. A Ca^{2+}-ATPase-rich fraction was eluted between 200-300 mM imidazole when a 10-500 mM imidazole gradient was applied. To protect the ATPase activity of the solubilized Ca^{2+}-ATPase, 1 mM Ca^{2+} and 0.8 mg/ml phospholipids were added during washing and elution steps. Using the 6xHis C-terminal tag, a fraction containing 10 to 20% Ca^{2+}-ATPase of the total proteins was collected whereas the 6xHis N-terminal tag led to a fraction in which Ca^{2+}-ATPase represents 30-40% of the total proteins. In these fractions, ATPase activity of the recombinant Ca^{2+}-ATPase as a function of Ca^{2+} and ATP concentrations was found very similar to Ca^{2+}-ATPase purified from skeletal muscle.

After NTA chromatography the Ca^{2+}-ATPase can be further purified on a DEAE column. When a fraction from a NTA column containing the Ca^{2+}-ATPase with a C-terminal tag is loaded on a DEAE column and eluted by a 50-250 mM NaCl gradient, the Ca^{2+}-ATPase appears in a fraction giving a single band on SDS-PAGE stained with Coomassie blue. Such a complete purification cannot be achieved with a Ca^{2+}-ATPase carrying a N-terminal tag.

CEA, DBCM/SBPM et URA CNRS 2096, Centre d'Etudes de Saclay, F-91191 Gif sur Yvette Cedex

6-17

PROTEINS ARE QUANTUM MECHANICAL AND NONLINEAR MACHINES, L. Cruzeiro-Hansson

Macroscopic machines are made of components which interact with each other as rigid bodies. The view that the components of a protein machine also behave in the same manner is sustained by the observation of different conformations of the proteins at different stages along their cycles which show that α-helices, β-strands and other protein domains move as a whole. However, a closer look reveals that conformational changes are not achieved by rigid body motion and that a hierarchy of processes, occuring at different timescales, is involved. The focus here is the energy transduction of the chemical energy released in the hydrolysis of ATP into different types of work, such as muscle contraction, active transport, protein folding and DNA repair. A model for the very short timescale (ps-ns) after the chemical reaction is presented according to which the energy is initially stored in the amide I vibration of the peptide groups. The model predicts that at biological temperature the energy can travel from the active site to other regions of the protein in a stochastic way. The integration of this short time process into the full protein cycle will be sketched.

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6-18

TEMPERATURE-INDUCED STRUCTURAL CHANGES IN THYLAKOID MEMBRANES AND LAMELLAR MACROAGGREGATES OF LHCII. Z. Cseh[1,2], I. Simidjiev[2], G. Garab[2] and E. Papp[1].

Chloroplast thylakoid membranes and lamellar aggregates of the light harvesting Chl a/b complexes (LHCII) have been shown to be capable of undergoing light-induced reversible structural changes (Garab et al., 1988, Biochemistry 27, 2430.). These changes affect the photophysical pathways in the antenna system (Barzda et al.,1996, Biochemistry 35, 8981.), and are likely to be involved in protecting plants against excess radiation but its physical mechanism is yet to be clarified. In this work, we tested the hypothesis that the light-induced changes are due to thermooptic effect, similar to that in liquid crystals. We measured the temperature stability of different circular dichroism (CD) bands of different physical origins. These results showed that elevated temperature (40-50 °C) leads to a disassembly of macroaggregates, (loss of psi-type CD bands), but has no effect on the chromophore interactions of the complexes (excitonic CD bands were unaffected). These data are consistent with the hypothesis that dissipation of excess light, if it leads to the elevation of the local temperature in the macrodomains, can transiently lead to a „clear-up" in the chiral macroorganization of the chromophores. Model calculations show that, if the heat exchange between the medium and the macrodomains is not very fast, the absorbed light can induce a gradual disassembly of the macroorganization of the complexes.

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6-19

THE STRUCTURE OF THE OXGYEN-EVOLVING MANGANESE COMPLEX INVESTIGATED BY X-RAY ABSORPTION SPECTROSCOPY ON VECTORIALLY ORIENTED MEMBRANE SHEETS H. Dau*, H. Schiller, L. Iuzzolino, J. Dittmer, W. Dörner, V. A. Solè, W. Meyer-Klaucke, H. Nolting

Elucidation of the reaction mechanism involved in photosynthetic water oxidation is seriously hampered by the lack of sufficient structural information on its catalytic center, the tetra-nuclear PS-II manganese complex. Multilayers of thylakoid membrane sheets, each containing numerous PS II, with a preferential orientation of the membrane normal perpendicular to the plane of the flat support (usually a Mylar or Kapton foil) have been used for collection of XAS spectra at various angles between the x-ray electric field vector and the thylakoid membrane normal (x-ray absorption linear dichroism spectroscopy, XALDS) [1-3]. **(I)** Now we obtain more precise structural information by XALDS due to the following methodical improvements: precise determination of mosaic spread disorder by correct analysis of EPR-spectra, avoidance of radiation damage [6], precise normalization, a correct approach to describe the angle dependence of apparent coordination numbers, estimation of the influence of multiple scattering effects [6], resolution of 'sub-shells'. **(II)** Comparison of new XALD data on the structure of the PS II manganese with hypothetical structures obtained by a molecular modelling approach reveals that presently various structural models are equally likely to resemble the 'real' complex. **(III)** Use of EPR [7,8] and XALDS enables us to compare the structure of the PS-II manganese complex of higher plants (spinach, barley) and green algae (*Scenedesmus obliquus*, *Chlamydomonas reinhardtii*).

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6-20

MOLECULAR CHARACTERIZATION OF THE VESICULAR MONOAMINE TRANSPORTER. C. Sagné, M.F. Isambert, J. Vandekerckhove*, J.P. Henry & B. Gasnier.

Classical neurotransmitters are released from neuron terminals by exocytosis. Their storage into synaptic vesicles requires an active transport from the cytoplasm catalyzed by a H^+/neurotransmitter antiporter, which utilizes the H^+ electrochemical gradient created by an ATP-dependent H^+ pump. A tetrabenazine- and reserpine-sensitive vesicular monoamine transporter (VMAT) is responsible for the uptake of catecholamines and serotonin into secretory and synaptic vesicles. Two homologous VMAT cDNAs have been cloned and the VMAT-2 protein has been purified from bovine adrenal medulla. Ketanserin is another powerful inhibitor of VMAT which bind to the same site as tetrabenazine. A photoactivable derivative, 7-azido-8-iodoketanserin (AZIK) was used to map the inhibitor binding site on the protein. AZIK was shown to covalently label VMAT-2 and digestion with endoproteases V8 or Lys-C revealed that the label is restricted to a 7 kDa peptide. The digestion products were separated by electrophoresis, transferred to a PVDF membrane and sequenced. For both enzymes, the material co-migrating with the labelled peptide produced a sequence matching to the N-terminus of bVMAT2. Consistently, mutagenesis of the lysine-55 codon of the bVMAT-2 cDNA to glutamic acid was shown to prevent the formation of the 7 kDa labelled peptide upon Lys-C proteolysis, with a redistribution of the label in higher molecular weight digestion products. We conclude that the segment 2-55 of VMAT2 contain residues involved in the binding of ketanserin and, possibly, tetrabenazine.

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6-21

ABOUT THE ROLE OF 70 kDa POLYPEPTIDE IN THE FUNCTIONING OF PHOTOSYSTEM 1 CORE. I.M. Guseinova, S.Yu. Suleimanov and J.A. Aliev.

It is very important to elucidate the main polypeptide of PS 1 core with M_r 70 kDa in the functioning of photoactive pigment P700. According to the literature date 70 kDa polypeptide is carrier of photochemical active form of chlorophyll a at 700 *nm* and responsible for photoinduced signal at 700 *nm*. However, the data obtained by us show that after inhibitor protein synthesis effect in chloroplasts by lincomycin 70 kDa polypeptide is lost and the intensity of Chl *a*-protein of CP 1 on the electrophoretic map is strongly decreased. Absence of this polypeptide does not almost influence on photochemical activity of PS 1 reaction centre the treated samples, which are measured by two ways: 1) polarography method on photoreduction of MV from DCPIP; 2) photoinduced change of absorption at 700 *nm* (ΔA_{700}). In spite of that CP 1 and this apoprotein is strongly reduced by the inhibitor of protein synthesis, at the same time it does not influence on the photochemical activity of PS 1, it probably exists the electron flow from reaction centre of PS 1 through system of interchangeable carrier, which are cytochrome and plastocyanin. It is also possible that in this case other pigment molecules of reaction centres with less activity and differing from P700 enter to photochemical reactions. It is not also excepted that non-identified low molecular polypeptides of PS 1 core also take part in this process. The final decision of this question requires the further careful investigations.

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6-22

THE EFFECT OF DROUGHT ON NATIVE PIGMENT FORMS IN HIGHER PLANTS. M. Ismailov, I. Zulfugarov and J. Aliev.

The stability of pigment forms of plants connected with physiological adaptation of leaves to the level of drought was investigated. To study the effect of drought stress on native pigment forms of wheat seedlings, the method of derivative absorption spectroscopy was used. It was shown that drought treatments at 20°C invariably resulted in sharp bleaching of chlorophyll *a* form band at 682 *nm* (Chl 682) and bands of β-carotene molecule with maxima at 452 and 486 *nm*. Full recovery of above bands to normal level was observed when water-stressed plants grown at low visible irradiance were watered. We suggest that Chl 682 is associated with D1 key protein of reaction center of photosystem II (PS II) and β-carotene molecule is associated with lipid fraction of PS II. Recovery of these pigment forms is connected with ***de novo*** synthesis of D1 protein and β-carotene molecule in watered seedlings at low intensity light and it could act as a general adaptation mechanism for the plants in response to drought stress.

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6-23

THERMAL DENATURATION OF FLAVODOXIN FROM ANABOENA PCC 7119 FOLLOWED BY DIFFERENTIAL SCANNING CALORIMETRY. Sandhya Jain

A Differential scanning calorimetry study of the thermal denaturation of flavodoxin was carried out at several pH at different concentrations and scan rates. The calorimetric traces were found to be reversible and highly scan rate dependent. The scan rate dependence can be explained by assuming that thermal denaturation of flavodoxin takes place according to the kinetic scheme $Apo_n + FMN \rightleftharpoons Holo_n$. The equilibrium constant of the equilibria has been evaluated as a function of temperature at some chosen pHs. The denaturation parameters obtained for the holoenzyme have been compared with those previously obtained for the apo form [Genzor etal. (1996) Protein Science, 5, 1376]

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6-24

DIRECT ELECTRIC MEASUREMENTS ON A MONOLAYER OF ORIENTED PHOTOSYNTHETIC REACTION CENTERS

S. Keller and W. Leibl.

Primary reactions in photosynthesis imply a sequence of charge transfer steps which occur in a protein complex called reaction center (RC). The RCs are located in the periplasmic membrane and contain four bacteriochlorophyll, two bacteriopheophytin and two quinone molecules. It is know from the X-ray structure that these cofactors are arranged in two symmetrical branches. Each charge transfer step which has a component perpendicular to the plane of the membrane gives rise to a corresponding phase of the membrane potential and can therefore be detected with macroscopic electrodes. By measuring the membrane potential variation in time, the kinetics of the electrogenic charge transfer can be studied, and information on the distance between the co-factors can be obtained. The RCs are asymmetrically oriented in the membrane. this orientation is necessary for their function and leads to a vectorial transfer. In order to observe an electric signal in the photovoltage measurements the RCs have to be oriented. This orientation is achieve by adsorption on a polyester film or on a platinum electrode coated with lipids. We were able to measure kinetics in the time scale from microseconds to seconds. Chromatophores and isolated reaction centers of the purple bacteria *Rb sphaeroides* and *Rps viridis*, asymmetrically adsorbed on an electrode coated with a lipid monolayer, allowed the study of the protonation of the second quinone. Furthermore, interactions between the reaction center and bound or soluble co-factors have been investigated.

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6-25

ELECTRON TRANSFER STEPS IN PS I-TYPE REACTION CENTERS: A COMPARATIVE PHOTOELECTRIC STUDY. W.Leibl

Light-induced electron transfer reactions in the photosynthetic reaction centers of photosystem I (PS I) and of the related heliobacteria were studied by time-resolved photoelectric measurements in the picosecond and nanosecond time range. Multilayered thin films of membrane fragments were obtained by electrodeposition onto a platinum electrode. This system shows a high and stable degree of orientation allowing to follow the transfer of the electron from the primary electron donor (P) along the chain of electron acceptors ($A_0 \rightarrow A_1 \rightarrow F_X \rightarrow F_A \rightarrow F_B$ in PS I). The relative amplitudes of the electrogenic phases give information on the relative transmembrane distances between the cofactors involved in the multistep charge separation process. The results obtained on PS I from *Synechocystis* compare well with the structural model emerging from X-ray crystallography. The photoelectric response from *Heliobacillus mobilis* is distinctly different from the response observed in PS I. In particular, in heliobacteria the primary step of charge separation $P \rightarrow A_0$ is followed by an electrogenic event about 20 times slower than in PS I (700 ps instead of 30-40 ps) and with a two times larger relative amplitude. Interestingly, the amplitude of the 700 ps-phase in heliobacteria matches the sum of the relative amplitudes of the electron transfer steps $A_0 \rightarrow A_1$ and $A_1 \rightarrow F_X$ in PS I. These results and results obtained by other techniques suggest that the equivalent to the phylloquinone acceptor A_1 in PS I is probably missing in heliobacteria and that in this system electron transfer from the primary acceptor A_0 occurs directly to the iron-sulfur center F_X (see also Abstract by Brettel et al.).

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6-26

KINETICS AND ENERGETICS OF PHOTOCYCLE IN REACTION CENTER OF PHOTOSYNTHETIC BACTERIA. P. Maróti and Sz. Osváth.

Electron and proton transfers are fundamental processes required for the generation and interconversion of energy in biological systems. In photosynthetic bacteria, the primary events for the conversion of light into chemical energy occur in a membrane bound pigment-protein complex called the reaction center (RC), which couples electron and proton transfer across the bacterial membrane. The photocycle involves excitation by light, electron transfer to the secondary quinone, Q_B, cytochrome exchange, proton uptake by reduced Q_B upon second electron transfer and exchange of Q_BH_2 with exogenous quinone. Minimal kinetic model of photocycle including both quinone (Q-6) reduction at the secondary quinone binding site and (mammalian) cytochrome c oxidation at the cytochrome docking site of isolated RCs from photosynthetic purple bacteria *Rhodobacter sphaeroides* was elaborated and tested by cytochrome photo-oxidation under strong continuous illumination. The typical rate of photochemical excitation by a laser diode at 810 nm was 2.200 s^{-1} and the rates of stationary turnover of the RC (one half of that of cytochrome photo-oxidation) were 600 s^{-1} at pH 6 and 400 s^{-1} at pH 8. The rate of turnover showed strong pH-dependence indicating the contribution of different rate-limiting processes. The kinetic limitation of the photocycle was attributed to the turnover of the cytochrome c binding site (pH<6), light intensity and quinone-quinol exchange (6<pH<8) and proton coupled second electron transfer in the quinone acceptor complex (pH>8). The analysis of double reciprocal plot of the rate of turnover vs. light intensity has proved useful method to determine the light-independent (maximal) turnover rate of the reaction center (445 s^{-1} at pH 7.8).

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6-27

ELECTRON TRANSFER AT LOW TEMPERATURE : *CHROMATIUM TEPIDUM*, ANOTHER CASE OF SUDDEN BLOCKAGE AROUND 220 K. F. Drepper, T. Nozawa and P. Mathis.

The photosynthetic reaction center (RC) of the thermophilic purple bacterium *C. tepidum* has a bound tetraheme cytochrome (4H-cyt), with two high-potential hemes (Em above +350 mV) and two low-potential hemes (Em below +150 mV). Flash excitation of RC induces oxidation of the primary donor P by electron transfer to a chain of acceptors ; P^+ is re-reduced by the 4H-cyt. We studied the effect of temperature on the kinetics of that reaction by kinetic flash absorption in chromatophores when both high-potential hemes are reduced :

- the $t_{1/2}$ increases slightly between 294 ($t_{1/2}$ = 500 ns) and 217 K ($t_{1/2}$ = 1040 ns). An Arrhenius plot gives an activation energy of 5.2 kJ.mol^{-1};
- the fraction of P^+ which decays by this fast reaction decreases rather steeply around 230 K from nearly 100 % at 294 K to nearly 0 % below 190 K where P^+ decays slowly ($t_{1/2} \approx 2.5$ ms), probably by return of an electron from the Q_A or Q_B quinonic acceptors. It seems then that the 4H-cyt becomes non functional.

A similar behaviour has been found in two other species of purple bacteria : *Rps. viridis*, which also has a firmly bound 4H-cyt, and *Rb. sphaeroides*, for electron transfer from a monoheme cyt c_2, either spontaneously docked or cross-linked to the RC [Ortega and Mathis, Biochemistry, 1993, 32, 1141 ; Venturoli et al, Biochemistry, 1993, 32, 13245 ; Drepper et al, Biochemistry, 1997, 36, 1418]. This behaviour, which has been attributed to freezing of networks of water molecules [Ortega et al, FEBS Lett., 1997, 401, 153] thus appears to be fairly common for the reaction of cytochromes in photosynthetic RC [see also the work of Rubin et al, e.g. Photosynth. Res., 1989, 22, 219].

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6-28

KINETICS OF THE XANTHOPHYLL CYCLE. J. Sielewiesiuk and W.I. Gruszecki.

In our kinetic scheme of the xanthophyll cycle, we take into account the evidence that light-harvesting complex (LHCII) catalyses zeaxanthin epoxidation. In dark-adapted chloroplasts, all pool of the cycle xanthophylls consists of violaxanthin bounded to LHCII. Under illumination, some of the violaxanthin molecules acquire triplet excitation, undergo cis-trans isomerization and dissociate from the protein. LHCII becomes then energetically uncoupled from PSII reaction centre (RC). Both xanthophylls can be readsorbed to LHCII and can cause its recoupling to RC (following epoxidation in the case of zeaxanthin). At saturating illumination, some of LHCII complexes are phosphorylated and can not be readily recoupled to RC. In this pool of LHCII, the probability of triplet excitation of violaxanthin is at least 10 times higher than in those coupled to RC. Phosphorylated LHCII complexes form the pool of so-called available violaxanthin. Violaxanthin of these complexes is easily dissociated into lipid matrix, where it undergoes deepoxidation. The cycle is completed by zeaxanthin adsorption to LHCII and its epoxidation within the complex. In order to obtain the full conversion of violaxanthin into zeaxanthin, we have to suppose that zeaxanthin can be photo-dissociated from LHCII prior to its epoxidation. So, the light can influence the xanthophyll cycle reactions by: 1. Inducing LHCII phosphorylation, a co-operative effect. 2. Acidifying thylakoid lumenal space to the optimum pH of de-epoxidase, co-operative effect. 3. Photo-dissociation of zeaxanthin from LHCII resulting in the inhibition of epoxidation, linear effect.

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6-29

LES LIAISONS DITES RICHES EN ENERGIE. J. Tonnelat

On admet qu'une transformation peut se produire lorsque dF est négative, F étant par définition égale à U – T S. Dans le cas d'une réaction chimique bimoléculaire en milieu liquide dF = - RT lnK + ln (a' b'/a b). a, b, a', b' étant les concentrations des réactifs initiaux et finaux. Quand lnK est positif et très grand, dF est négative même lorsque le produit a·b est petit devant a'·b'. Quand dans un tel cas une liaison est rompue, on dit que la liaison est riche en énergie.

Les liaisons considérées comme riches en énergie sont des liaisons qui se rompent pour une valeur du produit des concentrations des réactifs de départ faible devant celle des substances obtenues.

F, bien qu'appelée l'énergie libre, *n'est pas une énergie* parce que S n'en est pas une. Il peut y avoir création d'entropie à partir de rien alors qu'on ne peut créer de l'énergie. Il n'existe aucun rapport nécessaire entre dF et la variation d'énergie interne dU de l'ensemble des réactifs. Il n'y aucune raison pour que l'énergie libérée par la réaction soit nécessairement très grande.

La définition de l'entropie présente de graves difficultés. Mais, quand il n'existe pas d'interactions à distance entre les réactifs, le sens du déroulement d'une réaction est déterminé par les probabilités relatives de l'état initial et de l'état final en raison des déplacements des molécules et de leurs échanges d'énergie incohérents résultant de l'agitation thermique. L'état le plus probable est celui qui peut être réalisé du plus grand nombre de façons par permutations des molécules de même espèce dans l'espace et sur les niveaux d'énergie. L'introduction des logarithmes du nombre de ces permutations, grandeurs physiques bien définies, permet d'obtenir une relation équivalente à la relation habituelle, mais dans laquelle F est remplacée par un nombre sans dimensions.

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6-30

THE ROLE OF CLORORESPIRATION IN PS II INACTIVATION IN *CHLORELLA* UNDER UNFAVORABLE CONDITIONS. Yu. Chemeris, P. Venediktov and A. Rubin.

PSI is known to be inactivated in nitrogen- or phosphorus-deficient *Chlorella*. It was previously shown that the inactivation is accompanied by an increase in the constant fluorescence yield, F_o, indicating the loss of the photochemical quenching of excitons by PS II. We investigated causes of the PS II inactivation and found that diverse conditions that led to the loss of the photochemical quenching of chlorophyll fluorescence (nitrogen deficiency, heterotrophic growth on glucose, or incubation at 40-43°C in darkness) also enhanced chlororespiration in *Chlorella*. The temperature dependence of the rate of PS II inactivation in the dark-incubated alga was found to be identical to that of chlororespiration rate. Both rates drastically increased above 39°C. Both PS II inactivation and the enhancement of chlororespiration were prevented under conditions that prevented the accumulation of carbohydrates in algal cells (unavailability of CO_2 for nitrogen-starving alga or inhibition of glucose metabolism by 2-desoxy-D-glucose, a nonmetabolizable analog of glucose). In the alga subjected to heat shock, PS II inactivation was promoted when the oxygen-dependent oxidation of plastoquinone was inhibited under anaerobic conditions or in the presence of salicylhydroxamate, an inhibitor of the chloroplast oxidase. Nitrogen starvation did not result in PS II inactivation in the strain of *Chlorella* that accumulates excessive photosynthates in cytoplasm in the form of lipids. It is concluded that the activity of PS II in *Chlorella* is regulated by the amount of carbohydrate metabolites in algal chloroplast. The inactivation of PS II prevents their excessive accumulations under conditions which are unfavorable for growth.

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6-31

FT-IR STUDY OF TYROSINE PHOSPHORYLATION IN WATER AND IN INCTACT CELLS. M.Vivona, A.M.Villa, R.A.Gambetta*, L.Pirola, and S.M.Doglia.

A new method is presented to study tyrosine phosphorylation in solution and in intact cells by Fourier Transform Infrared Spectroscopy (FT-IR). The FT-IR absorption spectra of tyrosine and phosphotyrosine, obtained in water solution by attenuated total reflection on a ZnSe horizontal window, display significant differences in the region of the phosphates modes from 1150 to 950 cm^{-1}. These spectral differences have allowed to monitor the dephosphorylation of phosphotyrosine at millimolar concentrations, catalysed in solution by alkaline phosphatases, in excellent agreement with standard optical methods. Similarly, serine and threonine and their phosphorylated forms have been investigated in the same spectral range. It has been found that the spectral response of the phosphorylated forms of the three aminoacids differs appreciably in the absorption region of the phosphate modes, a result that could be useful to monitor which aminoacid undergoes phosphorylation in a protein.

This study has been extended to the investigation of phosphorylation in intact living cells. The human carcinoma A431 cell line, a good model system for tyrosine phosphorylation, has been examined. After subtraction of the water spectrum, high quality FT-IR spectra have been obtained for confluent cells with absorption intensities of about ten milliabsorbances. Well reproducible FT-IR spectra have been found for A431 cells exposed to epidermal growth factor (EGF) and to the inhibitor AG213 at different concentrations (20-200μM). From the analysis of the spectra in the phosphates absorption region (1150 - 950 cm^{-1}) it has been possible to evaluate the extent of phosphorylation in tyrosine occurring in the presence of different effectors, in agreement with immunochemical Western blotting study. The FT-IR spectroscopy could then offer a reliable and non-destructive method to study the *in situ* activity of tyrosine kinase.

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6-32

DEPENDENCE OF THE BACTERIORHODOPSIN INTERMEDIATE SPECTRA ON pH. L. Zimányi, C. Gergely and G. Váró.

The photochemical cycle of bacteriorhodopsin, the light induced proton pump of Halobacterium salinarium has been investigated by measuring transient absorption spectra after laser flash excitation in the visible spectral range. Typically, 25-35 difference spectra were collected on a logarithmically equidistant time scale from approximately 200 ns to hundreds of milliseconds, from pH 4.5 to 9.0, every 0.5 pH unit. Singular value decomposition treatment of the data matrices yielded four spectrally distinguishable components, and the data were reproduced and noise-filtered accordingly. Kinetic information and previous knowledge of the photocycle tell, however, that five intermediates, K, L, M, N and O are generally required to account for the photocycle, and we have searched for the absorption spectra of these five intermediates. A Monte-Carlo based search algorithm was combined with our previously applied set of criteria regarding the shape of the intermediate spectra. The wide range of potential spectra at each pH was further narrowed by fitting the corresponding measured (mixture) difference spectra and selecting those pure intermediate spectra that yielded the best ten fits to the data. The remaining sets of potential intermediate spectra at each pH were similar enough to each other to justify averaging, thus providing a single solution at each pH. Comparison over the entire pH range has shown no systematic variation in the spectra of the K, L, N and O intermediates, therefore these were further averaged to yield a single, pH-independent set. There was a systematic, approximately 2 nm red shift of the M spectrum with increasing pH, which may be caused by the deprotonation of a yet unknown side chain with a pK close to 7.

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7-1

NEAR–FIELD OPTICAL MICROSCOPY. NEW CONFIGURATIONS LIMITING THE RISK OF ARTEFACTS, POTENTIAL APPLICATION TO BIOLOGY. D. Courjon, D. Van Labeke and C. Bainier.

Near–field optical microscopes can be divided into three families, the transmission microscopes or SNOM, the reflection microscopes called R–SNOM and the internal reflection microscopes known as STOM or PSTM. Although the working modes of these microscopes have been explained thoroughly for the last years, no microscope has really demonstrated a particular aptitude for biological applications. One of the main reasons is probably the impossibility of discriminating the "true" optical information from topography data. Moreover, some configurations known for their capacity of detecting weak index or faint topographical variations such as the Scanning Tunneling Optical Microscope (STOM) introduce spurious effects due to an anisotropical illumination.

The present tendency is the realization of isotropical illumination devices, combined with a faithful topographical detection system in order to minimize the optical and topographical artefacts. According to this philosophy, a new near–field microscope has been built in Besançon. Using an isotropical illumination device allowing a dark field illumination, the tip position is controlled by measuring the oscillation damping of a home-made tuning fork when approaching the object surface. Some preliminary results are described and commented. These local results will be followed by a survey of the recent developments of near–field optical microscopy in the framework of biological applications.

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7-2

4PI-CONFOCAL MICROSCOPY PROVIDES FUNDAMENTALLY IMPROVED, NEARLY ISOTROPIC THREE-DIMENSIONAL RESOLUTION. S.W. Hell, M. Schrader, M. Nagorni

The advantage of far-field microscopy over electron microscopy and scanning tip approaches is the possibility to produce three-dimensional images from the interior of translucent specimens. However, the resolution of conventional or confocal microscopes is limited to about 200 nm, and 500-800 nm in lateral and axial direction, respectively. 4Pi-confocal microscopy improves the resolution by simultaneously employing two lenses of high numerical aperture. In 4Pi-confocal microscopy of type A, two 1.4 NA oil immersion lenses are placed opposite to each other and coherently illuminated so that the two wavefronts add up to single wavefront. 4Pi-confocal microscopy is particularly efficient when used in a multiphoton fluorescence mode (1). Using a two-photon excitation wavelengths of 750-800 nm, 4Pi-confocal microscopy achieves a resolution of 200 nm and 140 nm, in lateral and axial direction. Besides improving the axial resolution four-to fivefold, 4Pi-confocal microscopy also provides a solution to the problem of non-isotropic 3D-resolution in far-field imaging. The almost spherical effective focus reproduces the object with higher fidelity. The improved axial separation also improves the possibility to recognise objects in lateral direction (2). Thus it becomes possible to entirely distinguish clustered 100 nm fluorescencent beads in three-dimensions. To our knowledge, 4Pi-confocal microscopy provides the highest 3D-resolution with light at present. As an example of biological imaging, the three-dimensional structure of the F-actin in mouse fibroblast cells is resolved with unprecedented resolution in three-dimensions.

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7-3

CONFOCAL RAMAN MICROSPECTROSCOPY AND IMAGING OF CELLS. C. Otto, N. M. Sijtsema, I. Segers-Nolten and J. Greve

A number of cells in the blood and tissue of human beings are capable of recognizing and phagocytosing virusses, bacteria and other body-strange invaders of the human body. Upon recognition of such particles a large number of processes occur ranging from intracellular signalling to large scale material transport. Also molecular systems are prepared to participate in their task in the destruction of invaders. We have studied the molecular processes taking place when a neutrophilic granulocytes recognizes a body-strange particle.

The technique that we used was confocal Raman microspectroscopy. With this technique it is possible to acquire the Raman scattering of a small volume, typically 1 μm^3, of a single, living cell. The Raman scattering contains information on the vibrational states of the molecules inside the sample volume and is as such helpful in the identification of the molecules. Next to this the Raman scattering process is sensitive to structural aspects of the molecules, such as their three-dimensional organization, ligand binding state, redox state, etc.

The neutrophilic granulocytes were activated with different species, resulting in different cellular reaction. Also cells were investigated from patients with Chronic Granulomatous Disease. A reasonable interpretation will be put forward in terms of a number of enzymatic processes that occur in the living neutrophilic granulocyte.

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7-4

USING ELECTRON CRYOMICROSCOPY AND 3D IMAGE RECONSTRUCTION TO UNDERSTAND HOW MOLECULAR MOTORS WORK: KINESIN-MICROTUBULE COMPLEXES. I. Arnal, RH Wade.

Kinesins are a superfamily of motor proteins. They use ATP hydrolysis to fuel movement along microtubules and participate in many crucial phases of the eucaryotic cell cycle. Usually these motors are heterotetramers with globular motor domains on the two heavy chains. Most kinesins move towards the microtubule plus end, but some like ncd move the other way. Heavy chain dimers produced by over-expression are viable motors. We have used electron cryo-microscopy and three-dimensional reconstruction methods to investigate the effect of nucleotides on the structure of kinesin and ncd dimers attached to microtubules. The polarity of the 3D reconstructions can be determined for each individual microtubule and, in the presence a non-hydrolyzable ATP analogue, AMP-PNP, the motors have one unattached, and one attached head. The attached heads of kinesin and ncd appear to be similar and to interact with the same region of the plus end oriented subunit of the tubulin heterodimer. The free heads are quite different, suggesting that directionality could be determined by differences in the dimer conformations. The influence of the nucleotide state on the motors is under investigation.

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7-5

DIRECT OBSERVATION IN THE MILLISECOND TIME RANGE OF MEMBRANE EVENTS USING FLUORESCENCE VIDEOMICROSCOPY ACQUISITION SYSTEM. B. Gabriel and J. Teissié.

Interaction of propidium iodide and ethidium bromide with the membrane of electropermeabilized living cells is observed using a ultra-fast fluorescence image acquisition system. The computing process is linked to an ultra-low-light intensifying camera working with a very short time resolution (3.33 ms per image). We observed enhanced fluorescence of the dyes when associated with the electropermeabilized parts of the cell membrane. Images of the fluorescence interaction patterns of the two dyes digitized in single cell, reveal asymmetric local permeabilization of the membrane. For electric field intensities higher than a first threshold value, permeabilization is always observed on the anode-facing side of the cell. For electric field intensities over a second higher threshold value, the two electrode-facing hemispheres of the cell are permeabilized, the hemisphere facing the anode being most permeable. The asymmetric pattern of the dye interaction is neither dependent on the nature and the concentration of the dye nor on the ionic strength of the pulsing buffer or on the duration of the pulse. The field intensity determines the fraction of the membrane in which permeabilization can occur by means of free diffusion-like processes. The level of permeabilization in this localized region is controlled by the duration of the pulse when one single pulse of ms time range is applied.

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7-6

THREE DIMENSIONAL DIC VIDEO MICROSCOPY AS A TOOL FOR INVESTIGATION MIGRATION OF NEUTROPHILS L. Köhler, N. Korb, M. Eberhardt, H. Gruler

The active locomotion of cells is an important process in morphogenesis, in wound healing, in the defense system, etc. Human granulocytes are investigated which form the first defense line against invading microorganisms. The machinery of these amoeboid cells is very well investigated by using trajectories (=the position of the center of mass of the whole cell as a function of time). The chemotactic and galvanotactic response can be described as an automatic controller with an input stage, an amplifier and a feedback loop. The purpose of this work was to investigate the involved cellular compartments and their interplay by means of the DIC video microscopy. The small inherent depth field of DIC is used to acquire optical sections of the cell and then a three dimensional picture of the cell is reconstructed using computer based image processing tools (NIH image). A complete volume picture can be acquired in less than 1 s for e.g. 15 cellular sections with a z-spacing of 1 μm. A three dimensional movie is obtained if the procedure is repeated at different time increments. The time resolution is good enough to investigate the cellular machinery since the involved characteristic times are 8 [1] and 60 [2] s. The advantage of DIC is that the membranes show a strong contrast. The leading front as the cellular motor starts with the plasma membrane followed by a region with only little contrast (crosslinked actin filaments). Our observations of cells migrating on a flat glass surface are in accordance with those migrating in a narrow glass tube (plasma membrane followed by a structureless region) [3]. The advantage of our technique is that shape changes of the plasma membrane can be visualized as a function of time. This method is not restricted to granulocytes. It can be applied to other cell types like fibroblasts, osteoblasts, melanocytes, neural crest cells, etc.

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7-7

ATOMIC FORCE MICROSCOPY PROVIDES A MESOSCOPIC VIEW OF LIPID HETEROGENEOUS DISTRIBUTION IN SUPPORTED MODEL SYSTEMS. V. Vié, N. Van Mau, E. Lesniewska, J-P. Goudonnet, F. Heitz and C. Le Grimellec.

The average size and geometry of fluid and gel domains in phase-separated mixtures of supported phospholipids remains difficult to determine by conventional methods. Similarly, one has to use indirect methods based on the visualization of marker proteins, like cholera toxin (CTX), in order to determine the distribution of glycosphingolipids between fluid and gel phospholipid domains. The present experiments were undertaken to evaluate the possibilities of the atomic force microscope (AFM) for the imaging of phospholipid mixtures. Both Langmuir-Blodget monolayers and bilayers of DOPC/ DPPC (1/1) containing or not the ganglioside GM1 (1-5%) have been formed on freshly cleaved mica. Samples were examined in air (monolayers) or under liquid (20 mM NaCl, pH 7.5) with a Nanoscope III AFM. For DOPC/DPPC mixtures, domains of sizes ranging from ~50 nm to several μm in diameter were easily visualized due to the difference in height between fluid and gel phase lipids. Differences in the viscoelastic properties of the two phases were also imaged. Upon GM1 addition, the ganglioside was preferentially incorporated in the gel phase where bumps ~ 30 nm diameter appeared. These data demonstrate that AFM is a very powerfull tool for studing lipids domains. Because we imaged 2D arrays of CTX at the surface of DOPC/DPPC devoid of GM1, our data further indicate that one has to be carefull when using the cholera toxin as a probe of GM1 distribution in lipids.

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7-8

INTERNALISATION OF A STABLE OLIGONUCLEOTIDE HAIRPIN FOLLOWED BY MICROSPECTROFRET. M. Réfrégiers, B. Jollès, L. Chinsky and A. Laigle.

- Given the stability of our desoxyribonucleotidic sequence in serum, its cellular uptake has been monitored and its subcellular localisation determined. Single fluorescent labeling is not sufficient as you cannot know whether you observe the fluorophore alone or still grafted to the oligonucleotide. Therefore, intramolecular fluorescence energy transfer studies of double stained oligonucleotide were performed inside single cells by using confocal microspectrofluorimetry method.
- The oligonucleotide was labeled either i) with rhodamine at its 5' end, or ii) with fluorescein at its 3' end, or iii) at both ends for fluorescence energy transfer studies. They were added to the K562 cells suspension (10^6 cells/ml) at the therapeutic concentration of 5 10^{-6} M. Subcellular microvolumes (few cubic micrometers) were analysed with the confocal microspectrofluorometer developed in the laboratory. The 457.9 nm line of an Argon Laser was used for excitation.
- We have compared the cellular internalisation of the two mono labeled oligonucleotides with the double grafted one and with the internalisation of free fluorophores. Two different penetration pathways were determined for the double stained dodecamer.
- This method was found to be useful for the observation of the fate of short oligonucleotides in cell.

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7-9

MOBILITY OF β-ENOLASE IN THE CYTOSOL OF MUSCLE CELLS. EFFECT OF CREATINE KINASE. M. Arrio-Dupont[1], A. Keller[2], M. Vacher[1], G. Foucault[1] and S. Cribier[3].

An interaction between MM creatine kinase and β-enolase has been observed *in vitro* (T. Merkulova et al., Biochem. J. in press). The aim of this work was to detect the interaction *in vivo*. Myotubes were obtained from culture of satellite cells. MM creatine kinase and β-enolase were labeled with FITC (about 1 dye molecule per subunit). The mobility of each protein was determined in myotubes by modulated fringe pattern photobleaching. On the time scale of the experiment, 30 to 50% of creatine kinase was immobile, whereas β-enolase was totally mobile. To see if β-enolase could bind the immobile creatine kinase *in vivo*, FITC-enolase was co-injected with unlabeled creatine kinase. Under these conditions, the β-enolase was still completely mobile, which means that no interaction with creatine kinase was observed. This was confirmed by studies on skinned muscle fibers. Glycerol skinned fibers were incubated with FITC-β-enolase or FITC-creatine kinase, or with various mixtures of free creatine kinase and labeled β-enolase. Creatine kinase is localized on the M-line and near the Z-line. No bound β-enolase was observed, not even in the presence of a large amount of creatine kinase. These observations indicate that if the two enzymes interact in some glycolytic complex, the interactions are too weak to be seen by FRAP technique.

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7-10

PH GRADIENT CHARACTERIZATION IN MULTIDRUG-RESISTANT TUMOR CELLS BY MEANS OF DUAL RATIOMETRIC FLUORESCENT PROBES AND CONFOCAL SPECTRAL IMAGING R. Belhoussine, H. Morjani and M. Manfait.

Tumor cells have been shown to have an abnormally acidic cytoplasmic pH (Simon & Schinler, *PNAS*, 1994, 91, 3497). Here we report on the use of fluorescein and tetramethylrhodamine conjugates to dextran (DFR) and carboxy-SNARF1-AM to determine pH in acidic compartments and cytoplasm respectively of human sensitive and multidrug-resistant MCF7 tumor cells.

The microspectrofluorometer M51 (Dilor, Lille, France) was used to record confocal fluorescence spectral images from living cells. DFR are non-diffusible pH probes (MW 70.000). Thus after 18hrs incubation of the cells in presence of DFR at 1mg/ml at 37°C, a large amount of conjugates were introduced into cellular compartments by lengthy pinocytosis. Emission spectral images (λexc : 488nm) of conjugates display a pH-dependent band at 520nm (fluorescein) and pH-independent band at 580nm (tetramethylrhodamine). The ester-linked carboxy-SNARF1-AM (1μM) enters the cell passively where the esters are hydrolyzed by esterases in the cytoplam (45min at 37°C). Then, all areas of the cytoplasm demontrated an isotropic distribution of the dye. pH-dependent emission spectra (λexc : 514nm) of carboxy-SNARF1 (580nm and 640nm) lead to determine the cytosolic pH. For in situ calibration, each cell line was exposed to a buffer at a known pH containing nigerecin/high K^+ (20μM/150mM). Results show that cellular compartments in sensitive tumor cells (MCF7S) fail to acidify (pH gradient : 0.56). In marked distinction, drug-resistant tumor cells (MCF7R) appear normal (pH gradient : 1.19). Thus, drug sensitivity could be proposed of an acidification defect within vesicles of the recycling secretory pathways.

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7-11

WHY GOLD ISLAND FILMS ARE APPROPRIATE SUBSTRATES FOR RED AND NEAR-INFRARED MICRO-FT-SERS CELLULAR INVESTIGATION OF ANTICANCER AGENTS? **A. Beljebbar, G.D. Sockalingum, H. Morjani, J.F. Angiboust, M. Manfait.**

FT-SERS has been mainly reported on roughened electrode surfaces and colloids using 1064 nm excitation. In this work, we propose to evaluate gold island films as potential substrates for investigating the molecular and cellular interaction of antitumour agents using red and near-infrared sources. The films were prepared by thermally evaporating the metal onto cleaned glass slides in a vacuum of $5x10^{-5}$ torr. FT-SERS activity of gold films could be easily controlled by varying the experimental parameters such as thickness, deposition rate, temperature and pressure. All these contribute to optimise the efficacy of these substrates with a good SERS enhancement level. Our results show that strongly enhanced Raman spectra can be obtained from molecules adsorbed on gold island films using red and near-infrared excitations and that the enhancement level was comparable to that obtained with silver colloids. Furthermore, we also demonstrated the ease with which micro-FT-SERS data could be obtained concerning the interaction of differentiating agents such as dimethylcrocetin and all-*trans* retinoic acid in human cancer cell lines as K562 and HL60. This approach is more straightforward since with these substrates one benefits from the dominant long-range electromagnetic enhancement. Thus, there is no risk of disrupting the cellular integrity compared to colloids where the introduction of the micelles inside the living cell is very hard to reproduce and might perturb its integrity. In addition, their simplicity of production, relatively good reproducibility and stability make them more attractive for such studies.

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7-12

ROLE OF THE VACUOLAR H^+-ATP IN DAUNORUBICIN DISTRIBUTION BY THE MULTIDRUG-RESISTANT ASSOCIATED PROTEIN: A MICROSPECTROFLUOROMETRIC ANALYSIS. Z. Benderra, H. Morjani and M. Manfait.

Some multidrug-resistant cell lines efflux anticancer drugs but do not overexpress the well known P-glycoprotein pump or Pgp. A 190kDa or multidrug-resistant associated protein (MRP) have been identified and a pathway of drug extrusion which involves the concentration of the drug into cytoplasmic vesicles followed by an exocytotic process was proposed. In our study, we propose to study daunorubicin subcellular distribution in presence of a H^+-ATP pump inhibitor 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD) in myeloblastic K562 cells, MRP (K562-HHT30) and Pgp (K562-HHT300) overexpressing cells resistant to respectively 30ng/ml and 300ng/ml of homoharringtonine. Nucleo-cytoplasmic distribution of daunorubicin was carried out using scanning confocal microspectrofluorometry. This technique leads to characterise drug containing vesicles and especially nuclear accumulation of the anthracycline. Data show that daunorubicin sequestration in cytoplasmic structures occurs in both resistant cell lines. Nuclear accumulation of daunorubicin (1μM, 2hrs at 37°C) was as following : K562: 195μM ; K562-HHT30: 94μM ; K562-HHT300: 30μM. NBD at 0.5 μM concentration, was able to increase nuclear accumulation of daunorubicin in K562-HHT30 cells (185μM) but not in K562-HHT300. This NBD dose could reverse drug resistance only in the K562-HHT30 cell line.

In summary, data presented here demonstrates clearly that even if vesicular sequestration can happen in cells overexpressing MRP and Pgp proteins, only the MRP protein is able to extrude the drug through intracellular vesicles and exocytosis.

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7-13

CRYSTALLOGRAPHIC ANALYSIS OF FREEZE-FRACTURE ELECTRON MICROGRAPHS: APPLICATION TO THE STRUCTURE DETERMINATION OF CUBIC LIPID SYSTEMS.

H. Delacroix, A. Gulik and T. Gulik-Krzywicki.

The cubic phases of bicontinuous and micellar lipid-water systems have been studied by freeze-fracture electron microscopy. The preservation of the sample structure following cryofixation was verified by low temperature X-ray diffraction. Different types of fracture planes were identified; all display highly ordered two-dimensional domains, each subdivided into sub-domains related to each other by displacements and rotations related to the symmetry of the space group. The images were filtered using cross-correlation averaging techniques and the filtered images were compared to the corresponding planar sections of the electron density maps. Several conclusions were drawn:
(1) When well cryofixed, as assessed by low temperature X-ray diffraction, the structure of the sample was well preserved in the replicas.
(2) The symmetry of the space group was faithfully reflected in the electron microscope images.
(3) The crystallographic orientations of the most frequently identified fracture planes coincided with those of the most intense X-ray reflections indicating that the fracture propagates, preferentially, along well-defined planar paraffinic layers.
(4) When different structural models are compatible with X-ray diffraction data, it is possible to determine the correct model by comparing the filtered images with sections of the corresponding electron density maps.
(5) This approach constitutes a new and powerful tool of general interest for the structural study at low resolution of three-dimensionally ordered specimens like lipid-protein crystals.

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7-14

QUANTITATIVE ANALYSIS OF CELL MIGRATION AND NUCLEUS MOVEMENTS BASED ON OPTICAL FLOW COMPUTATION OF DIGITIZED MICROSCOPIC IMAGES.

A. Doisy[1,2], F. Germain[3], P. Tracqui[3], M. Robert-Nicoud[1] and X. Ronot[1,2]

Cell migration plays a crucial role in several physiological and pathological processes like embryogenesis, wound healing or tumour invasion. We propose a quantitative analysis of cell migration and associated nucleus movements based on optical flow techniques, i.e. on the calculation of the velocity field in sequential digital images. Our quantification is based on the estimation of the six parameter set of an affine motion model. The constant part of the affine model characterizes the nucleus and cell translation, while the linear part figures their rotation and/or deformation respectively. Experimentally, a model of *in vitro* wound healing has been developped as a simple way to get a directional cell migration toward the wound centre. Images were acquired with a phase contrast and fluorescence microscope. Nuclei movements were tracked concomittantly using a non perturbant nuclear DNA fluorescent staining.
First, our motion analysis provides a precise determination of the migration velocity of the cells at different times during the wound healing process. Second, a refined quantification of the variation of the affine model parameters with time indicates the existence of a time-lag between nucleus and cell displacement, as well as the nucleus rotation during cell migration. This analysis of cell and nuclei movements should be applicable to quantify the effect of chemiotactic factors (growth factors ,...) and various substrata (collageneous or fibronectine coated surfaces, ...). In addition, this approach should allow a direct analysis and quantification of the correlation between cell movement and nucleus DNA reorganisation.

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7-15

CONFOCAL SPECTRAL IMAGING ANALYSIS OF MITOXANTRONE INTERACTIONS IN LIVING CELLS.

A. Feofanov*, S. Charonov**, I. Kudelina*, F. Fleury**, M. Manfait**, and I. Nabiev**

Mitoxantrone (Novatrone™), a synthetic anthraquinone drug, shows considerable activity against a wide variety of animal tumor models as well as in clinical treatment of acute nonlymphocytic leukemia, advanced breast cancer and non-Hodkin's lymphomas. The exact mechanisms responsible for the antitumor action of mitoxantrone (mito) has not been elucidated. It may be one or several mechanisms related to the ability of mito to bind to DNA, cytoskeleton proteins and/or to produce cytotoxic metabolites. Here we apply the confocal spectral imaging (CSI) technique to direct monitoring of the localization and interactions of the mito within living cells. We are starting from the detail study of the environmental factors and molecular interactions affecting the fluorescence of mito. The considerable distortion of mito fluorescence revealed in this work demonstrates that *in vitro* modeling of drug-target interactions is an important primary step on going to *in situ* study of the drug uptake, disposition and interactions. It predetermines the correct applications of CSI technique to the drug analysis in living cells. Basing on the results of *in vitro* modeling several states and interactions of mito within K562 cells were identified by CSI technique, namely, mito in aqueous environment, mito bound to hydrophobic cellular structures, as well as mito bound to nucleic acids. The naphtoquinoxaline metabolite of mito was also detected in the drug-treated cells. The basis set of reference spectra being well suitable to describe the state and interactions of mito was successfully approved to characterize accumulation and localization of mito in the K562 cells.

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7-16

LOCALIZED 3D MOBILITY MEASURED BY SCANNING MICROPHOTOLYSIS.

U. Kubitscheck, P. Wedekind, and R. Peters

Scanning Microphotolysis, a recently developed family of photobleaching and photoactivation techniques, was extended to three dimensional (3D) diffusion by means of single or two photon excitation. While operating a confocal microscope in the line scan mode the laser beam power could be switched during scanning between high photolysing and low monitoring levels at pixel accuracy using an accousto-optical modulator. The number, position, and length of line segments to be photolysed could freely be determined. The minimal photolysis volume was slightly larger than optical resolution in all three spatial directions. The temporal resolution was ≥0.5 ms. A general theoretical method for the evaluation of 3D diffusion measurements based on numerical simulation using threefold time-step splitting was developed and extensively characterized. The method could be applied to any photobleaching geometry, comprised photobleaching by both pulse or continuous illumination, and took the convolution of the photolysed pattern with the imaging microscope point spread function into account. Great care was taken to define the required simulation parameters like discretisation step sizes, and signal-to-noise ratios for which correct and unique results for the fitting parameters would be obtained. Experimental and theoretical procedures were verified by measurements on solutions of B-phycoerythrin in glycerol-water mixtures. The obtained diffusion coefficients aggreed well with expectation.

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7-17

VOLTAGE-SENSITIVE DYES: SPECTRAL RESPONSE OF ANNELLATED HEMICYANINES. B. Kuhn, P. Fromherz.

A novel class of hemicyanine dyes has been synthetized in which no free CC single bonds or CC double bonds affect the fluorescence [1]. We have studied the modulation of the fluorescence of these "annines" by the membrane voltage in Retzius neurons of the leech and for comparison also of well known hemicyanines such as RH421 and di4ANEPPS. In an improvement of a previous technique [2] we were able to record complete two-dimensional spectra of excitation and emission under the control of whole-cell patch-clamp. Two-dimensional spectra of voltage sensitivity were determined. The dye ANNINE-V1 had a sensitivity of about 30%/100 mV. The spectral change of this dye can be assigned to a pure electrochromic effect. A disadvantage of the novel dyes with respect to application in optical recording in neurons is their low solubility. It is difficult to stain the cells.

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7-18

ORIENTATION OF A HEMICYANINE DYE IN LECITHIN MEMBRANE ON SILICON. A. Lambacher, P. Fromherz.

Hemicyanine dyes are used as molecular probes of voltage transients. Their spectral sensitivity has been studied in lipid membranes [1]. The mechanism of voltage-sensitivity is unclear. The orientation of the chromophore with respect to the transmembrane electrical field is a crucial parameter to understand direct and indirect effects of voltage. We used the interaction of dyes with the standing modes of light in front of silicon to determine the orientation in a bilayer of lecithin. We used microscopic steps of silicon dioxide as spacers from the reflecting surface of silicon. They were coated with the stained bilayer. The fluorescence intensity was measured as a function of the thickness of the spacer. We fitted the intensities by an optical theory which takes into account the interference of the exciting light and of the emitted light [2]. As a reference the cyanine dye dil was studied with a transition dipole parallel to the membrane. In the case of the hemicyanine di-8-ANEPPS we found that its transition moment is not oriented normal to the membrane as expected from its structure. The data can be described by a random orientation of the transition moment with respect to the membrane normal.

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7-19

INTRACELLULAR PROBING OF THE MOLECULAR INTERACTIONS OF MITOXANTRONE BY RAMAN AND SURFACE-ENHANCED RAMAN CONFOCAL IMAGING. I. Chourpa, H. Morjani, S. Charonov and M. Manfait.

Mitoxantrone is a highly potent anticancer drug currently used in the clinical trials of non-Hodgkin's lymphomas, acute myeloid leukemias, and advanced breast cancer. To study the molecular interactions of this drug within living K562 human erythroleukemia cells (established from a patient with chronic myelogenous leukemia), we have employed Raman and surface-enhanced Raman (SER) confocal imaging. This work is based on our previous resonance Raman and SER investigations dealing with mitoxantrone-DNA complexes modelised in aqueous buffer solutions. A comparative resonance Raman and SER study of mitoxantrone-DNA model complexes demonstrated that the adsorption of the drug/DNA complex on the silver hydrosol surface does not induce detectable perturbations of the molecular interactions within the complex. The spectral data indicated that an interaction involves preferential intercalation of ring A and, in part, ring B of the chromophore inside the DNA double-stranded helix. This model has been supported by data from Raman and SER confocal microspectroscopy studies with living K562 cancer cells treated with mitoxantrone (1μM for 1h) and incubated with silver colloids. In order to obtain statistically valuable spectral data characteristic of different cellular compartments, we have applied spectral imaging analysis. Sets of the spatially resolved (1 μm resolution) Raman and SER spectra have been recorded using step-by-step scanning of the cellular compartments. Then spectral images were generated to display local distribution of the characteristic Raman features of mitoxantrone.

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7-20

ACTION OF HYDROSTATIC AND OSMOTIC PRESSURES ON LIVING CELLS. P. Mentré, G. Hui Bon Hoa, O. Zatsepina and P. Debey.

High hydrostatic pressures increase the degree of hydration of the macromolecules (i.e. the amount of water directly associated to their surface, generally referred to as *bound water*), while osmotic pressures dehydrate macromolecules. Changes in hydration affect both the conformation of macromolecules and their interactions. Moreover, as bound water has a reduced solving power, an increase (decrease) of bound water induces a decrease (increase) of free ions concentration.

We applied here hydrostatic and osmotic shocks on living cells (mouse early one-cell embryos in a physiological culture medium and somatic cells in culture) in an attempt to analyze how hydration (and/or related changes of free ions concentrations) affect macromolecular associations in cell compartments. Cells were analyzed by immunofluorescent labelling of major nucleolar proteins and electron microscopy. Free ions were detected at the electron microscopy level by the pyroantimonate method.

Our results show that cells are still viable after short (15-20 min) exposure to hydrostatic pressures <1300 bars or low osmotic (20 times dilution of isotonic medium) and that both treatments have different effects on the nucleolus, which is the most affected subnuclear compartment. Under hydrostatic pressures ≥ 1300 bars the nucleolar ultrastructure becomes rather homogenous and the usually recognizable subcompartments disappear. On the other hand hypotonic shocks induce a reversible dispersion of nucleolar proteins.

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7-21

SPECTROSCOPY AND EPIFLUORESCENCE OF MIXTURES OF POLYENE ANTIBIOTICS WITH DLPC MLV. J. Milhaud and A. Blume.

The interaction of amphotericin B (AmB)with multilamellar vesicles of dilauroylphosphatidylcholine (DLPC MLV) shows, by circular dichroism, the formation of two successive bound AmB states with the stoichiometry DLPC/AmB = 350±50. Similar results were reported with nystatin (Ny)[1]. Association complexes which could satisfy such a stoichiometry were inspected by epifluorescence after labelling the membranes by octadecylrhodamine B. Superstructures following from aggregation and fusion of MLV and consequently from an overcoming of the repulsive hydration force were observed.

A dehydrating effect of the antibiotic on DLPC membranes as a possible cause of these superstructures was explored by FT-IR study of Ny-containing D_2O(or H_2O)/CCl_4/DLPC reverse micelles. The Ny-induced modifications of the contours of the OD (or OH) water stretching bands and the DLPC C=O stretching bands, indicate a release of DLPC-bonded water.

From these results, the binding of both antibiotics is thought taking place at the interface of DLPC membranes through H-bonds involving the antibiotic OH groups.

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7-22

MICRO-MANIPULATION OF SINGLE DNA MOLECULE. A. MIZUNO, S. KATSURA, H. YASUDA, Y. MATSUZAWA, R. ISHII, K. HIRANO, A. YAMAGUCHI, H. IMAYOU and Y. MATSUI

An accurate manipulation of a single DNA molecule will support genome analysis, such as mapping and sequencing of genome DNA. This paper describes several key techniques for the manipulation, such as transportation and stretching of single DNA molecule, and localization of enzymatic activity in a limited area to promote chemical reactions in molecular basis.

(1)Manipulation of large DNA molecules based on conformation change (globule formation) in DNA molecules. Because large DNA molecules, such as chromosomal DNAs, are very fragile, it has been difficult to manipulate large single DNA molecules. We developed a novel technique for the manipulation using conformation change of large DNA molecules from random coil to globule state. It was demonstrated experimentally that the DNAs in globular state were tolerant for sharing stress. We can recover a single chromosomal DNA in globular state into a glass capillary in a solution.

(2)Transportation of single DNA molecule was achieved in frozen sample by the area which confined a DNA molecule.

(3)The local temperature control technique by laser irradiation was also applied to localize restriction enzyme activity. It was demonstrated experimentally that yeast chromosomal DNAs were cut within 10 μm area around the laser focal spot.

(4)Fundamental study on mapping of genes on chromosomal DNA was carried out based on fluorescent observation of a single stretched DNA molecule which was denatured and hybridized with complementary fragments. In this study, tandem ligated lambda phage DNA molecules were used.

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7-23

NILE RED AS INDICATOR OF PHOSPHOLIPID CONTAINING ORGANELLES IN MULTIDRUG RESISTANT CANCER CELLS : A SCANNING MICROSPECTROFLUOROMETRIC APPROACH. H. Morjani, R. Belhoussine and M. Manfait.

Multidrug-resistant cancer cells have been shown to have an abnormally accumulation and distribution of phospholipids in organelles (Lavie *et al.*, J. Biol. Chem., 1996, 271, 19530). In this study, we propose the use of nile red to probe phospholipid containing organelles in sensitive and multidrug-resistant cancer cells. Phospholipid containing organelles in sensitive and multidrug-resistant cancer cells have been identified by monitoring emission spectra of nile red (NR). In these compartments, increased NR accumulation induced an increase of the gold-yellow/red (580nm / 630nm) emission ratio intensity. Thus the accumulation of NR in phospholipids containing organelles was apparaised by the determination of the contribution of the gold-yellow emission intensity (I_{580nm}/I_{630nm}) in each emission spectrum, using laser scanning confocal microspectrofluorometry. An argon laser excitation line was used (488nm). In organelles of multidrug-resistant MCF7R and HL60R, I_{580nm}/I_{630nm} emission ratio is significantly higher (0.96 ± 0.25 and 1.16 ± 0.20) than the values (0.54 ± 0.10 and 0.75 ± 0.15) from MCF7S and HL60S sensitive cells. This result is interpreted as a more important accumulation of NR in cytoplasmic structures of resistant cells, which present probably abnormally accumulation of phospholipids (Beers, J. Biol. Chem., 1996, 271, 14361). Thus, the profile of phospholipids distribution in cellular organelles induces a shift from NR emitting in the red region (sensitive cells) to NR emitting in the gold-yellow when associated to high level and/or particular distribution of phospholipids (resistant cells).

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7-24

SIGNAL PROPAGATION IN RAT DENDRITE OBSERVED BY OPTICAL RECORDING W. Novak, P. Fromherz.

We studied the invasion of electrical signals from the soma into the dendrite of neurons from the hippocampus of rat using a voltage-sensitive dye. We mapped the time resolved voltage-pattern along a linear dendrite of 500 µm length in a single shot. The approach had two advantages as compared to a previous study [1]: The geometry of the dendrite was well defined. (ii) The combination of several records to attain a voltage-map - i.e. any effect of phototoxicity - was avoided. The dendrites were grown on straight lanes of laminin. They were stained with the novel voltage-sensitive dye BNBIQ using micellar staining [1]. A linear array of 100 photodiodes was used for detection with a resolution of 5 µm and 0.4 ms [2]. We compared the invasion of an action potential with the spread of transient hyperpolarization. The hyperpolarization was damped effectively, whereas the action potential appeared even at the tip of the dendrite. These results are the basis to study the modulation of signal propagation by the neuronal activity itself.

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7-25

HYBRIDIZATION OF NUCLEIC ACID PROBES IN LIVING CELLS: AN *IN SITU* STUDY BY FLUORESCENCE CORRELATION SPECTROSCOPY (FCS). S. Paillasson, X. Ronot, and M. Robert-Nicoud.

Fluorescent *in situ* hybridization techniques are widely used to detect nucleic acid targets in a sequence-specific manner. In most cases, however, these techniques can only be applied on fixed samples. The "anti-sense strategy", which uses specific oligonucleotides to inhibit the expression of a particular gene via sequence specific interactions, is based on the implicit assumption that hybridization also occurs in living cells. The potentialities of using nucleic acid probes for the localization of specific RNAs in living cell by fluorescence microscopy has not been fully investigated yet. Recent studies in our laboratory have shown that sequence specific hybridization of such probes is possible under certain conditions (Paillasson, 1997).
In this context, the aim of the present work was to investigate the processus of hybridization in living cells, to follow the kinetics of hybridization and to measure *in situ* the quantity of hybrids formed. Fluorescence Correlation Spectroscopy (FCS) has been widely used to follow the kinetics of assembly of macromolecules in solution. This technique is very sensitive and can measure macromolecular interactions in very small volumes.
The potential use of FCS to measure the binding of a fluorescent probe to his target inside living cells was investigated. Fluorescently labeled oligonucleotides specific for the 28S rRNA were introduced into cells by transient permeabilization. FCS measurements showed that : i) specific hybridization does take place in the nucleus of living cells, ii) about 30% of the fluorescence detected over the cell nucleus is due to probes hybridized to their targets after 90 minutes, and iii) no secondary interaction of the probe with other cell components can be detected.
These first results demonstrate the potential of Fluorescence Correlation Spectroscopy for investigating macromolecular interactions within living cells, and for assessing the degree of binding of fluorescent probes to their targets. The technique can also be used to measure the mobility of probes in different cell compartments. It therefore provides unique tools for investigating the dynamic properties of macromolecules in their natural environment.
Reference : Paillasson S., Van De Corput M., Dirks R.W., Tanke H.J., Robert-Nicoud M., Ronot X. (1997) *Exp. Cell Res.* 231, 226-233.

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7-26

AUTOMATIC SEGMENTATION OF CYTOLOGICAL IMAGES WITH A VIEW TO ANALYSE THE CELLULAR SOCIOLOGY OF CULTURED CELLS BY TOPOGRAPHY. J. Palmari, P.M. Martin and C. Dussert.

Segmentation is a very critical and difficult step in the analysis of cytological images. We present an image processing method applicable to stained cells or nuclei, imaged by optical microscopy, in which we use filtering algorithms (to increase the contrast between nuclei and background) and mathematical morphology (to preserve cell topography). A particular feature of our method is its ability to process the images of densely packed nuclei or badly stained cells. The aim of this method is twofold : (i) to compute for each cell various parameters related to its densitometry, texture, morphology; these data are then analysed by multiparametric satistical methods. (ii) to record the position of all of the cells with a view to study their topographical behaviour by means of a graph, the minimal spanning tree (MST).
The method is used to analyse the proliferation of the breast cancer cell line MCF-7 under estrogenic treatments. The computation of 15 parameters related to the densitometry and texture of the chromatin of Feulgen stained cells, allows discrimination of treated and untreated cells by multiparametric analysis. It is shown that cell treatments induce profound changes in populations distributions.
It is assumed that such a topographical analysis together with analysis of individual cells provides information about interactions and control processes between cells.

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7-27

SIGNAL PROPAGATION IN LEECH NEURITE OBSERVED BY OPTICAL RECORDING A. Prinz, C. Müller, P. Fromherz.

We studied the invasion of action potentials from the soma into the neurite of leech neurons in culture using a voltage-sensitive dye. In particular we asked the question: Does the invasion depend on the activity itself ? We improved the technique of optical recording as used in previous studies [1, 2] by projecting a linear neurite of 500 µm length onto a linear array of 100 recording photodiodes. So we were able to measure in a single shot the complete time-resolved pattern of voltage at a resolution of 5 µm and 0.4 ms. We found that that the invasion of the action potential was not changed even after a second of continuous firing.
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7-28

CHARACTERISATION OF WHEAT GRAIN BY FLUORESCENCE SPECTRAL IMAGING AND PRINCIPAL COMPONENT ANALYSIS. A. Saadi, S. Sharonov, and M. Manfait.

Phenolics are the most autofluorescent materials in cereal grains. To characterise them in wheat grains we have developed a method which permits, using microspectral imaging technique and PCA , to recover their space distribution and quantify their relative concentration. An XY set of fluorescence emission (excitation at 365 nm, and emission between 380-680 nm) spectra was recorded on transversal and longitudinal cuts of the grain by using the M51 scanning microspectrofluorometer. Each spectrum S_i was then considered as a combination of principal spectral components (C_j): $S_i = \sum A_{ij} \cdot C_j$; where A_{ij} is the contribution of j spectral component in the i spectrum. The principal components were obtained by the following two-step PCA procedure: firstly, spectra with maximal distance from each other are found, then these are corrected to avoid meaningless negative values of the contribution coefficients. These corrected spectra could then be used as model components which can be approximated to the real phenolic materials of the wheat grain. Three principal spectral parameters indicative of phenolics in the specific region of wheat section were identified as containing the features characteristic of certain phenolic acids (ferulic and p-coumaric acids). The spatial organisation of each spectral component in the wheat sections can be obtained onto reconstituted surface on the basis of integrated intensities of spectra.This method has then been extended to study wheat flours. We found that the spectral model previously determined can be used as a meaningful indicator of the undesirable tissues in order to estimate the aleurone contamination in wheat grains and flours.

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7-29

SIMULTANEOUS ANALYSIS OF INTRACELLULAR FREE CALCIUM AND EXOCYTOSIS DYNAMICS AS STUDIED BY UV CONFOCAL LASER MICROSPECTROFLUOROMETRY. M. Pereira, S. Sebille, J-M. Millot, M. Arnaud, A-M. Delabroise, J. Jacquot and M. Manfait.

Numerous inflammation mediators have been reported to stimulate mucus secretion from airway secretory cells. Moreover, intracellular free calcium ($[Ca^{++}]_i$) plays a central role in the exocytosis process of secretory cells. We report here the simultaneous analysis of both $[Ca^{++}]_i$ and secretory granules exocytosis following a histamine exposure. Using UV confocal laser microspectrofluorometry, dynamic changes in $[Ca^{++}]_i$ from single living cells were determined after Indo-1 staining. In addition, the secretory granules exocytosis was observed by the decrease of quinacrine emission intensity. The co-staining of both probes allowed to obtain emission fluorescence spectra which have then been analysed as a linear combination of three reference spectra, recorded *in situ*: Ca-free Indo-1, Ca-bound Indo-1 and quinacrine. This method was applied in the case of exposure of human tracheal gland cells to histamine (100 µM) which induces a transient $[Ca^{++}]_i$ increase ($\Delta[Ca^{++}]_i$ =240 ± 120 nM) and secretory granules exocytosis for 95% of cells. The consequence of varying concentrations of extracellular magnesium (0.4 to 10 mM) has been then investigated. First, the cells percentage which observed a $[Ca^{++}]_i$ rise decreased for high extracellular Mg^{++} (95% at 0.5 mM Mg and 75% at 10 mM Mg). Furthermore, the decrease of quinacrine fluorescence emission, one hour after histamine exposure, was lower in the presence of high extracellular magnesium (80% for 0.5 mM Mg and 30% for 10 mM Mg). In conclusion, important Mg concentrations modulate both the $[Ca^{++}]_i$ rise and the exocytocis process in human tracheal gland cells.

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7-30

ELECTRON TOMOGRAPHY RECONSTRUCTION OF PLASTIC-EMBEDDED ORGANELLES. B. Shillito, A. J. Koster, J. Walz and W. Baumeister.

Structural investigations on the chitin secretion system of a deepsea tubeworm, Riftia pachyptila, are presented. In this organism, 300 nm cup-shaped organelles are the sites of synthesis of large (50 nm) crystalline chitin microfibrils of the rare β form. Electron tomographic reconstructions of plastic-embedded cup-shaped structures were carried out, using 93 projections distributed over a +/- 70° angular range with a 0.66 nm pixel size. Moreover, the shrinkage profile of the plastic sections was measured to determine the required imaging conditions. The entire data collection was carried out automatically, and under cryo-conditions. Automation resulted in an efficient use of irradiation dose ; 96 % of the total dose is required for the data itself, only 4 % is used for the required compensation of focus change and image shift. The results are presented as 3 nm tick slices through the 3-D reconstruction. In these slices, structures that are overlapping in a conventional 2D projection of the initial thin section are visualized separately. Also, these thin slices through the 3-D reconstruction show the fine contours of the cone-shaped nascent microfibril tip.

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7-31

DETERMINING VIRUS STRUCTURE BY POLARIZED RAMAN MICROSPECTROSCOPY: SUBUNIT AND SIDE CHAIN ORIENTATIONS IN THE *Ff* FILAMENTOUS VIRUSES. Stacy A. Overman, Masamichi Tsuboi and George J. Thomas, Jr.

The *Ff* filamentous viruses (*fd, f1, M13*) are long and thin particles (~890 × 6 nm) consisting of a single-stranded DNA loop that is sheathed by ~2700 copies of a small α-helical subunit. These supramolecular viral assemblies are not amenable to high-resolution structure determination by either X-ray crystallography or multidimensional NMR spectroscopy. However, polarized Raman microspectroscopy of oriented fibers of the filamentous viruses is capable of providing important structural details, including the orientations of coat protein side chains and main chain with respect to the filament axis. Interpretation of the polarized Raman spectra for this purpose requires definitive band assignments, as well as knowledge of the local Raman tensors that correspond to vibrations of the targeted protein subgroups. Using Raman tensors determined for the amide I main-chain vibration and for key Raman markers of the tryptophan (W26) and tyrosine (Y21 and Y24) residues, we have determined the orientations of these key side chains in the native *Ff* virion. The results provide a basis for visualizing virion architecture and evaluating previously proposed assembly models. The methods developed here should be applicable to other biological supramolecular assemblies. [Supported by NIH Grant GM50776.]

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7-32

BIOPHYSICAL APPLICATIONS OF A FLUORESCENCE LIFETIME IMAGING MICROSCOPE.

A.J.W.G. Visser, J. Goedhart, A. van Hoek and T.W.J. Gadella Jr.

Fluorescence lifetime imaging microscopy (FLIM) is a technique which is able to directly acquire images representing the spatial distribution of nanosecond fluorescence lifetimes of an object in a fluorescence microscope. Our frequency-domain microscope employs periodically modulated excitation light, synchronous modulation of the amplification stage of a microchannel plate image intensifier (up to 120 MHz) and subsequent recording of the digital images with a CCD camera. We describe the frequency-domain FLIM instrumentation, the post acquisition digital image analysis for reconstructing fluorescence lifetime images, and applications of FLIM in cell biology. The following examples will be given. Fluorescence lifetime shortening of the flavoprotein lipoamide dehydrogenase induced by addition of a quenching inhibitor is demonstrated in an array of microcuvettes. This experiment exemplifies the substantial flavin autofluorescence which is often present in all living cells. FLIM of the calcium indicator Calcium Green is shown in hepatocytes in which the calcium levels are modulated (and distinct from control experiments) when cells are exposed to a tumour promotor. Fluorescence resonance energy transfer between phospholipids equipped with donor and acceptor fluorophores is monitored by FLIM revealing lipid-lipid interactions in 3T3 cells. FLIM also provides a new lifetime imaging contrast as shown in the visualisation of the *in situ* binding of fluorescent NOD factors to *Vicia* root hairs.

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8-1

D. Chatenay.

No abstract available.

8-2

MOLECULAR REACTIONS OF PROTEINS MONITORED BY TIME-RESOLVED FTIR SPECTROSCOPY.

Klaus Gerwert.

Time-resolved FTIR spectroscopy is recently established as new tool to monitor the dynamic behaviour of protein-side groups, the protein-backbone and prosthetic groups at the atomic level (1). Step scan FTIR spectroscopy resolve the intramolecular reactions with 30 ns timeresolution (2). In combination with molecular-biology techniques providing site specific mutated proteins absorbance bands can uneqivocally be assigned to specific molecular groups. This approach is applied to contribute to the understanding of the molecular reaction mechanisms of:

-the lightdriven proton pump bacteriorhodopsin (3,4)
-the bacterial photosynthetic reaction center (5,6)
-the Ca ATPase (7)
-the h ras protein p21 (8)
-the folding of cytochrom-c

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8-3

Native proteins and their conformational changes imaged with subnanometer resolution using an atomic force microscope

Daniel J. Müller & Andreas Engel, Biozentrum, Basel, Switzerland.

Bacteriorhodopsin from *Halobacterium salinarium*, head-tail connectors from phage ø29, hexagonally packed intermediate (HPI) layer from *Deinoccocus radiodurans*, and OmpF porin from *E. coli* were imaged with an atomic force microscope in buffer solution. A resolution of up to 0.8 nm allowed structural differences of individual proteins to be detected. Reversible structural changes of the cytoplasmic surface of purple membrane have been induced by changing the force applied to the scanning stylus: donut shaped bacteriorhodopsin trimers transformed into a structure with three pronounced protrusions, when the force was reduced from 300 pN to 100 pN. Similar to this, the thinner end of phage ø29 connectors were pushed onto the protein surface at forces between 50 and 100 pN. Furthermore, individual pores of the inner surface of the HPI layer were observed to switch from an 'open' to a 'closed' state. Together, the structural changes of proteins monitored under physiological conditions suggest that the direct observation of function-related conformational changes of biomolecules with the atomic force microscope is feasible. This is demonstrated on the extracellular surface of OmpF porin which undergoes pH-dependent reversible conformational changes. This may represent a protective mechanism that closes the channels at low pH.

8-4

FÖRSTER-TYPE RESONANCE ENERGY TRANSFER MEASUREMENTS IN DIGITAL FLUORESCENCE MICROSCOPY

P. Nagy, G. Vámosi and J. Szöllősi

Since the formulation of the Singer-Nicholson membrane model, one of the most important refinements in our knowledge about biological membranes is the understanding the role of protein-protein interactions. Since the Förster-type energy transfer rate is inversely proportional to the sixth power of the distance between the energy donor and acceptor, it is a suitable technique to assess proximity relationships between fluorescently labeled membrane proteins. Flow cytometry can measure the energy transfer on a cell-by-cell basis. If we want to resolve differences within single cells, we have to make use of fluorescence microscopy.

Photobleaching energy transfer (pbFRET) measurements have already been used for such purposes. One important premise in pbFRET measurements is that the photobleaching kinetics of the donor is only altered by energy transfer to the acceptor. In our hands this may not always be the case, e.g. with tightly associating receptors. To get around this problem, we developed a fluorescence microscopic approach. It is based on the measurement of three different fluorescence intensities (fluorescein, rhodamine and energy transfer "channel"). Since the spectrum of autofluorescence is cell independent, we can determine the ratio of autofluorescence at different wavelengths. If we measure the autofluorescence of labeled cells in UV, we can calculate the autofluorescence contribution in the above three channels. Considering the spill-over between different channels, we were able to calculate the energy transfer efficiency. To get reliable results, we had to increase the pixel size compared to pbFRET measurements, but this approach still enabled us to dissect intramembrane heterogeneity without the extreme sensitivity of photobleaching kinetics to environmental factors.

This work was supported by OTKA grant F022725.

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8-5

A MICRO PIPET BASED TECHNIQUE TO TEST BOND STRENGTHS — INDEPENDENT CALIBRATION.

D. A. Simson, F. Ziemann, M. Strigl and R. Merkel.

Experimental investigations of specific molecular adhesion are greatly hampered by the minuteness of the forces involved. Relevant forces are thought to range from 1 to 100 pN. Recently, a new micro pipet based technique has been presented (E. Evans, K. Ritchie and R. Merkel, Biophys. J. 68 (1995), 2580). Its main feature is the application of an osmotically preswoilen red blood cell as ultra soft, tuneable force transducer. It is pressurized by a micro pipet. This creates an uniform surface tension that resists elongation of the cell. Forces ranging from 0.1 to 1000 pN can be applied under physiological conditions. In order to check the assumptions of this technique we used a "magnetic tweezers" setup (F. Ziemann, J. Rädler and E. Sackmann, Biophys. J. 66 (1994), 2210). A paramagnetic latex bead (Dynabead M280) was glued onto a red blood cell that was aspirated by a micro pipet. A magnetic field gradient was produced by an electromagnet. The forces acting on the beads were calibrated using Stokes' law. Measurements of bead displacement versus electrical current were performed and allowed the experimental determination of the force-displacement-relation. The experimental results coincide with the theoretical predictions.

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8-6

GRATING COUPLERS AS INTEGRATED-OPTICAL SENSORS. APPLICATIONS IN MEMBRANE BIOPHYSICS.

R. Horváth, G. Meszéna and E. Papp.

Grating couplers on planar optical waveguides are a new type of chemical sensors. The waveguide is a thin film with higher refractive index than the surrounding medium. Illuminating the grating with laser beam at a certain angle the light can be coupled into the waveguide. The light in the waveguide propagates as normal modes. Different guided modes have different effective refractive indices (defined as the ratio of the phase velocities of light in vacuum and in the waveguide) and incoupling angle [1,2]. Adsorption and desorption of molecules on the waveguide surface, or other refractive index variations of the waveguide cover medium change the effective refractive index of the guided mode and so the incoupling angle [3]. Measuring this angle one can detect changes on the waveguide surface with high precision (Optical Waveguide Mode Spectroscopy, OWMS).

We have built an optical waveguide spectroscope with high angle resolution (6×10^{-6} degree). The details of the spectroscope will be presented. We measured the optical parameters of lipid bilayers and the changes in lipid bilayers in the presence of divalent and trivalent cations.

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8-7

DIRECT MEASUREMENT OF WEAK FORCES INVOLVED IN DNA BASES RECOGNITION

F. Pincet, E. Perez, L. Lebeau, C. Mioskowski and E. Evans

We have measured forces between adenine and thymine using functionnalized lipids. The adhesion between adenine and thymine is much larger than that between identical groups. At all distances, the forces are attractive, the dominant attraction being adenine-adenine at long distances and adenine-thymine at short distances. Thymine was also modified in order to prevent any pairing. By comparison with adenine-thymine, this provided a measurement of the range of the preferential interaction. The long-range attractions may involve collective effects which have been investigated.

We shall also introduce a new method to measure the energetics and range of weak biochemical bonds using functionalized vesicles. Large bilayer regions are held in molecular proximity by osmotic depletion forces acting upon two functionalized vesicles to enable rapid specific bonding. By fixing an electrical charge to the tethering site of the functional group on one surface, persistent adhesion of the vesicles subsequent to removal of the depletion stress is titrated against the range and magnitude of the clamped electrostatic potential of the opposite surface.

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8-8

BIOBEADS : AN EFFICIENT STRATEGY FOR TWO DIMENSIONAL CRYSTALLIZATION OF MEMBRANE PROTEINS.

J.L. Rigaud., G. Mosser, J.J. Lacapere, D. Levy, J.L. Ranck, P. Bron and O. Lambert.

This work establishes the potential of Bio-Beads as a more powerful and simple alternative to conventional dialysis for removing detergent and for obtaining 2D crystals useful for structure analysis by electron crystallography.

Kinetics and equilibrium aspects of removal of different detergents by adsorption onto hydrophobic Bio-Beads SM2 have been systematically investigated. This demonstrates a general use of this strategy for all kinds of detergents classically used in membrane protein solubilization, purification and reconstitution.

The method was further extended to the 2D crystallization of different classes of membrane proteins with radically different hydrophilic / lipophilic balances. Thus 2D crystals of Ca^{++} ATPase, cytochrome b6f, melibiose permease and F_0F_1 ATPases could be produced, with optical diffraction down to 20 Å in negative stain. In the case of cytochrome b6f, a projection map was calculated to 8 Å resolution. Different crystals of Ca^{++} ATPase (tubes, stacked crystalline bilayers) could be produced and crystallogenesis process analyzed in details, taking the additional advantage of Bio-Beads to be a convenient means for varying and controlling the rate of detergent removal.

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8-9

X-RAY ABSORPTION SPECTROSCOPY COUPLED TO SITE-DIRECTED MUTAGENESIS: AlcR PROTEIN LOCAL STRUCTURE. I. Ascone[a], F. Lenouvel[b], B. Felenbok[b].

The elucidation of the interactions between transcriptional factors and their DNA targets is a major challenge of present time biology. The AlcR activator, is a zinc DNA-binding protein and the metal content in the protein is in a 2:1 ratio. X-ray absorption spectroscopy is a technique of choice to obtain detailed information about the key metal atom and on the DNA-binding domain configuration. EXAFS analysis indicates Zn environment: four sulphur atoms bind one Zn atom at an average distance of 2.34 Å. Three mutants in the DNA-binding domain motif were studied by X-ray spectra to observe zinc sites as function of the amino acid mutation. In order to relate structural and biological data, the ability of the mutants to specifically bind AlcR DNA targets and to bind zinc was also tested and the results were correlated with zinc local structure. When one cysteine (Cys 49) ligand is mutated the following is observed: (a) the total loss of specific DNA-binding activity , (b) Zn content of protein decreases by two orders of magnitude and (c) XANES spectrum shape changes drastically. In contrast, mutation of one histidine (His10) does not change zinc environment. In conclusion, our data show that AlcR DNA-binding domain is a zinc binuclear cluster model in which two zinc atoms coordinate six sulphur ligands.

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8-10

EFFECT OF ENANTIOMERIC SALTS ON BOVINE SERUM ALBUMIN ADSORPTION ONTO $Si(Ti)O_2$ OXIDE FILMS. *In situ* MEASUREMENTS WITH OPTICAL WAVEGUIDE MODE SPECTROSCOPY (OWLS). V. Ball, J.J. Ramsden.

The pharmacological actions of two enantiomeric forms of a given drug are usually totally different in relation to stereospecific recognition by cell surface receptors. In the presence of adsorbed proteins or bulk dissolved proteins, the separation of enantiomeric amino acids or drugs can be performed by means of capillary electrophoresis (1). We are currently interested in the influence of different salts, on the protein adsorption process at solid-liquid interfaces. The recently introduced technique of OWLS (2) enables the kinetics of adsorption at an interface to be measured with excellent time and surface excess resolution. At constant ionic strength and pH, the two enantiomers of tartaric acid have been found to strongly modify the adsorption charateristics of BSA at pH 7.4. Adsorption is practically inhibited in presence of 10 mM D(-) tartaric acid whereas adsorption is strong but slow in presence of the L(+) enantiomer at the same concentration. The origin of this difference in adsorption behaviour is investigated by means of capillary electrophoresis (role of specific ion binding to the protein surface) and circular dichroïsm spectroscopy (role of structural modifications).

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8-11

HIGH HYDROSTATIC PRESSURE AS A TOOL TO STUDY PROTEIN STRUCTURE AND FUNCTION. C. Balny.

Recent decades have witnessed a growing interest on the part of researchers in introducing pressure as a variable acting on biosystems. It becomes clear that, along with such parameters as temperature and solvent conditions, pressure can be used for more detailed thermodynamic and kinetic description of bioprocesses and biosystems and regulation of their behavior. For example, in protein denaturation studies, high hydrostatic pressure provides unique information on unfolding mechanisms (notion of molten globule state).

On the other hand, the possibility of applying pressure in specific biotechnological areas, mainly for food processing, gives new applications of this parameter.

Due to technical progress, many instrumental problems have been solved, and today almost all the biophysical methods used routinely at atmospheric pressure have been adapted to high-pressure conditions (optical, vibrational or NMR spectroscopies, electrophoresis, fast kinetic methods, etc.).

Here we discuss recent selected examples of applying pressure in biochemical research :

- effects of pressure on noncovalent interactions (electrostatic, hydrogen bonds, hydrophobic interactions) ;
- effects of pressure on molecular structure and on inter-molecular interactions ;
- pressure effects on protein structure (stability - denaturation) ;
- combined effects of temperature and pressure ;
- pressure effects on enzymes reactions (volumes profiles and transition states) ;
- protein-nucleic acid interactions.

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8-12

FOURIER TRANSFORM NEAR-INFRARED SPECTROSCOPY IN THE STUDY OF ESCHERICHIA COLI BACTERIAL STRAINS (SUSCEPTIBLE AND RESISTANT TO β-LACTAMS). **W. Bouhedja, G. D. Sockalingum, P. Pina, P. Allouch, C. Bloy and M. Manfait.**

To-date, mid-IR spectroscopic techniques are widely used to characterise micro-organisms due to their highly specific signatures. In this work we have examined the possibility of using Fourier transform diffuse reflectance and transmission techniques in the near-infrared (FT-NIR) for the characterisation of bacterial strains. To do so, we used an *E. coli* C600 K12 susceptible strain and the transconjugant SHV2 (strain resistant to β-lactams) grown on a solid medium. Sampling was done either on glass slides or BaF2 windows respectively for these methods. To exploit the NIR data, second derivation was done on the mean triplicate spectra recorded with each technique. Cluster analysis, using average linkage between groups, was then performed on the data and results were represented as dendrograms. Our findings show that data obtained with the transmission method characterise very well the resistant from the susceptible strain, which is not the case with the diffuse reflectance method. This discrimination is based on the correlation similarity coefficient matrix, also known as Pearson's coefficient. Although no molecular information is available with the NIR techniques they present certain advantages when used for taxonomic ends. Indeed, they are more rapid (1 scan at 16 cm^{-1} resolution is enough) and circumvents the problems associated with spurious atmospheric bands, generally encountered in the mid-IR. This emerging technique is being extensively used for tackling industrial problems and can in the future find its application as an epidemiological tool complementary to current biochemical methods.

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8-13

MECHANISM OF LYSIS INDUCED BY (LEU, LYS) AMPHIPATHIC PEPTIDES: AN INVASION OF THE OUTER LEAFLET OF MEMBRANES WITHOUT SECONDARY STRUCTURE REQUIREMENTS. S. Castano, B. Desbat, M. Laguerre, J. Dufourcq.

(Leu, Lys) peptides from 5 to 15 residues, designed to generate ideally amphipathic α-helices, all display a strong hemolytic activity. All the peptides proved to be tensio-active and their affinity for the air/water interface and a DMPC monolayer increase in parallel with the peptide length. In order to better understand the mechanism of lysis and to establish relationships between structure-orientation and activity, the polarization modulation infra-red absorption reflexion spectroscopy (PMIRRAS) appears to be a powerful tool. Indeed, this technique allows to get *in-situ* structural and orientational informations about the peptides at the air/water interface as well as inserted into a DMPC monolayer. It results from this study that the longer peptides (≥12aa) are folded into pure α-helices as expected. But a structural transition occurs for the shorter ones (n≤9aa): they form intermolecular antiparallel β-sheets. Whatever their secondary structure and the nature of the interface (air/water or DMPC monolayer) the peptides are oriented flat at the interface. This is confirmed by computer modelisation. Then the lysis induced by peptides of the (Leu, Lys) series seems to be caused by invasion of the membranes by peptides which orientate flat on the outer leaflet. There is no strict secondary structure requirement: both the β-sheet and the α-helix structures generate active peptides.

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8-14

NOVEL APPROACHES FOR MAPPING THE CELL SURFACE.

J. Szöllősi, L. Mátyus and S. Damjanovich.

The "fluid mosaic" model of the cell membrane emphasizes the mobility and the autonomy of individual membrane proteins. Results of the membrane research of the last two decades, however, suggest that there is a high degree of lateral organization of plasma membranes. There is evidence that the non-stohastic co-distribution of membrane-bound proteins can play an essential role in signal transduction across the plasma membrane. In contrast to the immunological or biochemical techniques (e.g. coprecipitation, cocapping, chemical crosslinking) biophysical methods (such as fluorescence resonance energy transfer (FRET), atomic force microscopy) can be used to detect molecular clusters with little or no perturbation of the membrane structure. Although the application of FRET in biochemical research is not new, the latest developments in the flow cytometric and microscopic versions of FRET furnished useful information about the structural organization of receptor complexes in the plasma membranes of lymphoid cells on a nanometer scale (J. Immunol. '96. 157: 2939). Although the flow cytometric FRET measurement sacrifices spatial information on each cell, it provides FRET efficiency on a cell-by-cell basis for a large population of cells within a short period of time, characterizing subpopulations of the cells. FRET microscopy can reveal lateral heterogeneity in the surface of individual cells, and also cell-to-cell variations in molecular clustering although with much less statistical accuracy. A long range lateral distribution of labeled proteins in the plasma membrane can be detected by scanning atomic force microscopy (AFM). AFM provides a tool for investigating of the surface topography and suitable for imaging with high spatial resolution of living and fixed cells in their natural environment. Combination of these techniques allow us to investigate receptor clustering at molecular and supramolecular levels (PNAS, '95. 92:1122).

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8-15

PROTEIN CRYSTALLOGPAHY IN 2D: GRAZING INCIDENCE X-RAY DIFFRACTION FROM PURPLE MEMBRANE PATCHES AT THE AIR/WATER INTERFACE. A. Frenzen, M. Weygand, S. Verclas, N. A. Dencher, G. Büldt, P. Howes, K. Kjaer, M. Lösche

Purple membrane patches, the protein/lipid crystal sheets containing bacteriorhodopsin (BR) trimers have been deposited in monomolecular layers at aqueous buffer/air interfaces and characterized with grazing incidence x-ray diffraction (GIXD). While this method is well established for the determination of hexatic phases of amphiphilic monolayers at aqueous surfaces, its application to 2D protein crystals is still in its infancy. Our aim is to establish the technique as a new tool for structure determination in membrane proteins that are not amenable to 3D crystallization.

With native purple membranes deposited on 100 mM KCl subphases we have observed GIXD at various lateral pressures between $\pi \sim 7$ and 40 mN/m at room temperature. Diffraction peaks up to the 7th order [(h,k)=(4,3)] of the hexagonal unit cell have been measured, corresponding to a resolution of ~ 8 Å. At this resolution, the electron density map constructed with the phases known from electron microscopy[1] shows the typical annular arrangement of the α-helices in the BR trimer contained within the hexagonal unit cell. This result has been obtained with less than 10^{13} molecules (< 1 μg of protein) in the beam. Changes in the structure have been quantified upon compression of the 2D protein crystal at the interface, and from such measurements mechanical properties of the protein have been deduced. Delipidated purple membranes[2] at the buffer/air interface consist of less ordered 2D crystals and have been observed to diffract only up to the prominent (2,0) peak.

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8-16

MECHANICS AND KINETICS OF SINGLE RECEPTOR - LIGAND COMPLEXES MEASURED BY ATOMIC FORCE MICROSCOPY

J. Fritz and D. Anselmetti

Upon functionalizing an atomic force microscopy (AFM) cantilever, specific intermolecular recognition forces between a single molecule on the tip and its binding partner immobilized on a opposing surface can directly be measured. We investigate gold - mercapto, biotin - avidin, and cell aggregation factor complexes differing in their binding strength from several tenth of pN to several nN, as well as in their response to external forces. The latter can be related to different molecular elasticities of small molecules, globular proteins and chain-like proteoglycans.

Additionally, information on the binding kinetics between surface bound molecules can be obtained, if the rate constants of the binding are within the timescale of AFM experiments. In the case of a P-selectin - ligand binding, a time dependent decrease of the adhesion probability between AFM tip and surface could directly be related to the lifetime of a single ligand - receptor complex, which was found to be in the order of 0.2 sec.

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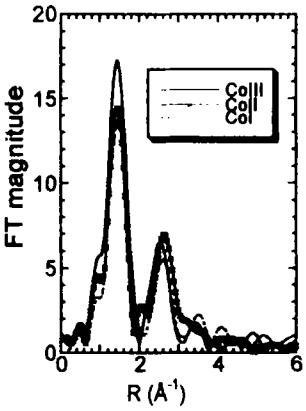
8-17

EXAFS STUDIES OF *IN-SITU* REDUCTION OF HYDROXOCOBALAMIN. M. Giorgetti, I. Ascone, M. Berrettoni, S. Zamponi, R. Marassi.

The cleavage of the Co-C bond during the reduction of Co(III) plays a key role in the biochemical activity of cobalamin compounds. We report the XAS studies at the cobalt K-edge for the progressive reduction cobalt cobalamins [Co(III)->Co(II)->Co(I)] obtained by *in situ* electrochemical methods. This experimental set up permits to record spectra in the same conditions thus making the comparison of different oxidation states more reliable. The spectra were recorded in fluorescence mode on a 2 mM solution of hydroxocobalamin with a 7 element Ge detector at D21 beam line of the DCI storage ring. The figure shows the Fourier transforms of the EXAFS signals for the progressive reduction of cobalt. The coordination number of metal decreases from Co(III) to Co(II) indicating the loss of a ligand. The simulated EXAFS spectra confirm the cleavage of one axial ligand. The progressive reduction to Co(I) does not affect the overall structure around the cobalt site.



Fourier transform of XAS signals.

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8-18

SERS STUDY OF THE INTERACTION OF THE PIRARUBICIN ANTIBIOTIC WITH PHOSPHOLIPID PLANAR BILAYERS USED AS SIMPLIFIED MEMBRANE MODEL

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Pirarubicin is an antibiotic used in chemotherapy. For many reasons as fighting against a cell resistance appearing during treatment, it is essential to understand the antibiotic-membrane interaction. In order to have information onto the crossing of the membrane by pirarubicin, we studied by Surface-enhanced Raman scattering (SERS) the orientation of this molecule into planar zwitterionic and anionic phospholipid bilayers. This technique consists of transferring different bilayers by a Langmuir-Blodegtt technique onto prisms previously coated with a thin silver layer, essential to increase the Raman signal. This spectroscopy having a very short range of detection, the molecules close to silver and thus really in contact with lipids are only observed. A preliminary surface pressure study enabled to determine the percentage of pirarubicin in interaction with phospholipids. Thus it has been shown that the presence of anionic phospholipids into the monolayer does not increase the percentage of pirarubicin, whereas it is positively charged, at the interface. This surprising result could be due to a different orientation of the antibiotic into the monolayer in the absence or the presence of anionic phospholipids. Using SERS we showed that pirarubicin is able to cross a pure phospholipid bilayer to come close to silver. Its orientation into the bilayer depends on the pirarubicin/phospholipid ratio, the charge of phospholipids and the mode of preparation of the bilayer. Two major orientations were found: pirarubicin is perpendicular to silver and thus embedded into phospholipids, or lying on silver and adsorbed on the polar head groups of phospholipids. This last orientation was found in two cases. i/ In the presence of anionic phospholipids in the bilayer: positively charged pirarubicin is adsorbed on negative polar head groups and forms a screen, hindering a deeper penetration of other molecules (this result agrees with the hypothesis proposed after the surface pressure study). ii/ In the presence of a high percentage of pirarubicin in the bilayer: excess of pirarubicin is ejected out of the bilayer and lyes on silver. We see here that surface pressure technique and SERS are two complementary methods for the study of membrane-exogenous molecules interactions.

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8-19

BIOLUMINESCENT MULTIENZYMIC TOXICITY TESTS: METHODS, PROBLEMS AND ADVANTAGES. V.A.Kratasyuk.

Bioluminescent Multienzymic Toxicity Test (BMTT) has been suggested. It consists of 4 bioluminescent systems: luminous bacteria, alcohol dehydrogenase, trypsin, NADH:FMN-oxidoreductase - luciferase. The main principle of this methods is the correlation between the toxicity of a medium under study and the changes of parameters of bioluminescence in vivo and in vitro. This BMTT has been successfully applied for environmental monitoring in laboratory and natural ecosystems: in the model laboratory systems of microalgue Spirulina platensis and Dunaliella salina, for toxicity control of portable and natural water in Altai and Krasnoyarsk regions, for research of volatile excretions of plants in the Life-support systems et al. Multicomponent immobilized reagents for toxicity has been created. Bioluminescence assays combine the advantages of speed and sensitivity, wide range of linear response to analyte concentration. The problems and perspectives of bacterial bioluminescent toxicity biotests are discussed.

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8-20

DODECYL MALTOSIDE : LIPOSOME SOLUBILIZATION, MEMBRANE PROTEIN RECONSTITUTION AND 2D CRYSTALLIZATION. O. Lambert, J.L. Ranck and J.L. Rigaud.

Dodecyl maltoside is a non-ionic detergent which has been found suitable for the purification of many membrane proteins. However few informations are available on the quantitative characterization of solubilization and reconstitution processes using this detergent.

The solubilization of large unilamellar liposomes by DOM has been analyzed in details and related to the "three-stages" model describing the interactions of detergents with membranes. The data allow to define the different steps of solubilization process and to quantify the mixed bilayer - mixed micelle interconversion. Analysis by cryo-electron microscopy of the structures formed during liposome to micelle transition allows to characterize a "gel" or condensed phase.

The process of membrane protein reconstitution into liposomes using DOM was analyzed through a step by step procedure (Rigaud *et al*, 1995, BBA, **1231**, 223). Active proteoliposomes containing Ca^{++} ATPase, bacteriorhodopsin and/or FoF1 ATPases could be produced after complete detergent removal by SM2 Bio-Beads.

These informations were further applied to the detailed analysis of the 2D crystallization of membrane proteins isolated and purified in DOM. Different types of low resolution 2D crystals of the melibiose permease (coll. G. Leblanc) and the ADP-ATP carrier (coll. G. Brandolin) have been produced.

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8-21

TWO DIMENSIONAL CRYSTALLIZATION OF MEMBRANE PROTEINS ON PLANAR LIPID FILMS.
G.Mosser, D.Levy, J.L. Rigaud.

Two dimensional crystallization of protein anchored on lipid monolayers has been sucessfully used for the structure determination of soluble proteins. Here conditions for the application of this method to membrane proteins solubilized in detergent are described. The protein is injected into the subphase with the detergent concentration above the cmc to avoid the protein aggregation. Binding of the protein to the lipid film is mediated through specific lipid/protein interaction using Hist-tag protein and NTA-Ni lipid. Finally detergent is removed by hydrophobic adsorption onto polystyrene beads Bio-Beads SM2. The processes of protein adsorption and lipid film organization were studied by epifluorescence, film pressure measurements and electron microscopy. Following detergent removal a stable lipid bilayer containing proteins is formed probably in contact with the lipid interfacial monolayer. Results with the melibiose permease of *E.Coli* show a high density packing of the protein in this bilayer, a prerequisite for futher crystallization.

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8-22

TRANSVERSE DISPERSION COEFFICIENT OF DNA DURING GEL ELECTROPHORESIS BY FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING.
L. Meistermann, B. Tinland.

The understanding of the dynamics of DNA molecules undergoing constant gel electrophoresis is a problem of great interest. The most commonly measured quantity in electrophoresis experiments is the DNA velocity. Experimental results dealing with dispersion coefficient of DNA are scarce because they are difficult to determine precisely ; even if one can observe DNA chain under the microscope, the resolution of band broadening measurements is not better than 1 µm. This work determine for the first time a complete molecular length, gel concentration and field dependence of Dy, the transverse dispersion coefficient perpendicular to the field direction. Values of dispersion coefficients have been measured by combining an electrophoretic cell with a Fringe Recovery After Photobleaching (FRAP) setup. Our results are consistent with the model of Biased Reptation with Fluctuations (BRF) [1,2]. The data have been compared with recent experimental values [3,4] of Dx, the longitudinal dispersion coefficient parallel to the field direction. We found a good agreement with theoretical predictions [5], suggesting that the longitudinal and the transverse dispersions coefficients are of the same order and their ratio is a universal constant (Dx/Dy = 9/4).

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8-23

TWO-PHOTON ABSORPTION OF LASER RADIATION BIOLOGICAL ACTIVITY MOLECULES

Y. P.Meshalkin

This paper reports the first measurements of the two-photon absorption (TPA) cross section of biological activity molecules: adrenaline, novokaine, cordiamine and coffeine at the wavelength of the second harmonic of Nd:YAG laser. All the measurements were carried out by the method of a two-photon standard, which is ipsensitive to the parameters of the exciting radiation. Measurements involved a comparison of the two-photon-excited luminescence intensities of the investigated substance and of a standard, which was a substanse with a known TPA cross section. The standard was p-bis(o-methylstyril)benzene (MSB) made by Eastman-Kodak (USA).
The TPA cross section of novokaine is - $3{,}07\ 10^{-49}$, adrenaline - $3{,}22\ 10^{-52}$, coffeine - $5{,}36\ 10^{-53}$ and cordiamine - $1{,}0\ 10^{-53}$ cm^4 s / photon mol.

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8-24

RELAXATION CURRENTS OF TRANSPORT PROTEINS ADSORBED TO SUPPORTED MEMBRANES CAN BE ELICITED BY RAPID FLUID INJECTION.
J. Pintschovius, K. Fendler and E. Bamberg.

Self-assembled monolayers (SAM) of long-chain n-alkanethiols were prepared by incubation of gold electrodes in a thiol solution. In a second step the hydrophobic alkyl surface was covered with a lipid solution (PC in decane) in order to obtain a double-layer similar to a black lipid membrane (BLM). Features of particular importance are the high electrical capacitance and the low conductance, which make the system appropriate for the measurement of currents generated by ion translocating proteins capacitively coupled to the lipid surface of the electrode. Either purified membrane fragments or vesicles containing the protein can be irreversibly adsorbed.
In contrast to the BLM, the solid supported membranes (SSM) are of high mechanical stability, and are therefore suitable to be combined with rapid fluid injection techniques. The preparation turned out to be stable even under conditions of rapid fluid flow (bulk velocities 0,5m/s). Time-resolved measurements were primarily carried out on the Na^+/K^+-ATPase. Current transients generated by ATP concentration jumps showed a time-resolution of 10ms. The technique is of special interest for concentration jump experiments, where substrates are not available as photolytically cleavable (caged) compounds, e.g. Na^+ and K^+. Preliminary experiments have shown that the method is compatible with a variety of other primary active, secondary active, and passive transport proteins.
The use of arbitrary activating substrates, the broad spectrum of transporters, which can be studied, the large number of experiments, that can be performed on the same preparation under a large variety of conditions, and the low amount of protein required are main advantages.

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8-25

MASC : A CONTRAST VARIATION METHOD IN X-RAY BIOCRYSTALLOGRAPHY. M. Ramin, W. Shepard, R. Kahn and R. Fourme.

Multiple-wavelength Anomalous Solvent Contrast (MASC) is a new method to produce contrast variation in a macromolecular single crystal (the contrast is defined here as the difference between the average electronic density in the macromolecule and the uniform density of the solvent). In the MASC method, contrast variation is obtained by tuning the X-ray wavelength near the absorption edge of an anomalous scattering species which is randomly dispersed in the mother liquor (Fourme et al. J. Synchr. Rad. 2,36, 1995). As in the neutron contrast variation method, only low resolution reflections are measured (d>5Å) whereas they are generally forgotten in standard biocrystallography. In practice, data sets including all low resolution reflections, up to about d=5Å, are collected at several wavelengths using a synchrotron radiation source.

The primary application of MASC is the determination of the macromolecular envelope. Moduli of the envelope structure factors are extracted from the experimental multiple wavelength data. Then, phases must be estimated in order to calculate, via an inverse Fourier-transform, a model for the envelope. Data sets for three test proteins, with known structures, have been collected at the ESRF (Grenoble) and LURE (Orsay). Experimental and model moduli are in good agreement up to at least d=20 Å. Ordered anomalous scattering sites at the surface of the macromolecule have been detected for these three proteins, which may also help phasing by combining MAD and MASC methods. Procedures to estimate phases are in progress.

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8-26

ACTIN ADSORBED UNDER PHOSPHOLIPID LAYERS, A. RENAULT, F. AMBLARD, C. GICQUAUD and B. BERGE

Besides its well-known role in muscle contraction, actin filaments are involved in a number of fundamental cellular processes including motility, cell division, phagocytosis, organelle movement and cell adhesion. Using an in vitro system based on a lipid monolayer supported by an aqueous buffer subphase, one can study the actin-lipid interaction and its effect on polymerization.

These interactions with lipid molecules lead to a change in lipid conformation and, as a consequence, a paracrystalline sheet of actin is formed at the lipid-buffer interface. This sheet is formed by parallel actin filaments in register with a 37.5 nm period and a 7 nm diameter, as seen by transmission cryo-electron microscopy. This system was afterwards improved using lipid monolayers which induced the formation of a bidimensional actin crystal having a high intrinsic order.

By ellipsometry studies, we investigated the adsorption of actin under a phospholipid monolayer (70% DSPC + 30% Stearyl amine), under conditions which prevent bulk polymerization in the subphase. We observed that the difference of ellipsometric angles due to theadsorption of actin under the lipid monolayer, is in agreement with a thickness of 7 nm, typical for the diameter of actin filaments. The same samples were used to measure the macroscopic rigidity by using a modifiedversion of the torsion pendulum. We observed the film shear deformation induced by an external torque applied to a float with a magnetic field. We found that the monolayer is in a solid phasewith a shear elastic constant of $\mu = (20 \pm 5)$mN/m.

We are currently developing dark field microscopy in order to directly visualize native actin filaments.

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8-27

CHARACTERISATION OF *SACCHAROMYCES CEREVISIAE* STRAINS BY ATR-FTIR SPECTROSCOPY AND HIERARCHICAL CLUSTER ANALYSIS. G. D. Sockalingum, W. Bouhedja, A. Gainvors, A. Belarbi and M. Manfait.

The isolation of indigenous strains of *Saccharomyces cerevisiae* from Champagne grapes must and their identification using biochemical and molecular biology techniques remain effective but heavy. We propose in this work a new approach, by hyphenation of ATR-FTIR spectroscopy and cluster analysis, which can rapidly characterise and classify different strains of *Saccharomyces cerevisiae* (CB2, CB3, CB4, CB8 and CB16). Yeast cultures grown on a solid medium were carefully harvested and homogeneously spread on a zinc selenide ATR crystal. Cluster analysis was performed using the normalised (in the 1800-900 cm^{-1} region) FTIR spectra and the result represented with the help of a dendrogram. This calculation is based on the mean distance between different classes. The results we have obtained with this approach are in a good correlation with conventional methods, where the caryotypes of these strains have been determined using pulsed field electrophoresis. In fact, strains CB3 and CB8 with the same caryotype were found to be characterised by the same distance in the dendrogram while strains CB2, CB4 and CB16 having all different caryotypes, exhibit different distances in the dendrogram representation. This type of spectral signature, complementary to conventional methods, can help to rapidly identify an unknown strain of *Saccharomyces cerevisiae* via the use of a data bank. Due to its rapid and non-destructive nature, it can be extended to other fields of great interest to the biotechnology industry.

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8-28

THREE-DIMENSIONAL IMAGE RECONSTRUCTION OF NATURALLY ASSEMBLED COMPLEXES OF pre-mRNA AND SPLICING FACTORS BY AUTOMATED ELECTRON TOMOGRAPHY. R. Sperling, A. J. Koster, M. Angenitzki, Z. Berkovitch-Yellin, and J. Sperling.

Splicing of pre-mRNA is a fundamental step in the expression of almost every eukaryotic gene, as only correctly spliced mRNAs are exported to the cytoplasm to function in protein synthesis. Naturally assembled complexes of mammalian nuclear pre-mRNAs can be released from cells nuclei as large nuclear ribonucleoprotein (lnRNP) particles. These particles contain all U snRNPs required for pre-mRNA splicing and all known protein splicing factors. Automated electron tomography was employed for the three-dimensional image reconstruction of 16 individual negatively stained lnRNP particles. For each particle, a tilt series of 71 images was collected by direct digital recording on a CCD camera attached to a computer controlled TEM facility. The 3D images were reconstructed, and interactive computer graphics was employed for rendering and comparison of the models. The reconstructed 3D images show a compact structure composed of four major subunits connected to each other. Comparison of the reconstructed lnRNP particles revealed morphological similarity of the individual particles, as well as similarity in shape and size of the subunits. We thus propose a model for the packaging of nuclear pre-mRNAs in lnRNP particles where each substructure represents a functional unit, presumably a spliceosome. This model is compatible with the requirements for alternative splicing, and with the non-sequential order of intron removal in multi-intronic pre-mRNAs.

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8-29

BAND BROADENING OF DNA DURING GEL ELECTROPHORESIS MEASURED BY FRINGE RECOVERY AFTER PHOTOBLEACHING. B. Tinland and N. Pernodet.

Electrophoretic methods are widely used to separate different molecular weights of DNA before sequencing. Improving separation conditions by understanding underlying physics is of great interest in Human Genome analysis. If the field dependence of the electrophoretic mobility, which is the major limitation to the electrophoretic separation of long DNA fragments in gels, is the most commonly measured quantity and has to be known to achieve good separation of different lengths of DNA, the dispersion coefficient, reflecting band broadening, is an other one. Band broadening, mainly arising from the distribution of the DNA conformations, exists during gel electrophoresis lowering the resolution of the separation and leading in some cases to band disappearance. Theoretical models like Biased Reptation Model (BRM) [Lumpkin, Dejardin, Slater, Noolandi] and Biased Reptation model with Fluctuations (BRF) [Duke, Semenov, Viovy] have been developped recently. Besides expressions of the mobilities, they developped expressions for the longitudinal and transverse dispersion coefficients. Under certain conditions, these parameters can be quantitatively determined by combining an electrophoretic cell with a Fringe Recovery After Photobleaching setup (FRAP). We present measurements of longitudinal dispersion coefficients D_x (parallel to the field) of DNA in various agarose concentrations on a master curve. Variations of the longitudinal dispersion coefficient with the electric field, the chain length and the gel concentration agree well with the predictions of the BRF. Its allows the prediction of the D_x value, whatever the DNA / gel system is.

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8-30

2D PROTEIN CRYSTALS UNDER A LIPID MONOLAYER, STUDIES BY ELLIPSOMETRY, ELECTRON MICROSCOPY, SHEAR MEASUREMENT OF LATERAL CRYSTALLINE ORDER AND X-RAY GRAZING INCIDENCE.

C. Vénien-Bryan, B. Berge, P-F. Lenne, A. Renault, C. Zakri, F. Balavoine, A. Brisson, G. Grubel, J. Lal, P. Vignais, C. Mioskowski, J-F. Legrand

The limiting step in structural studies of 2-dimensional (2D) crystals of proteins bound to a lipid monolayer is the production of highly ordered crystals.
a) In order to understand and improve the mechanism of formation and growth of 2D crystals, we have monitored the process by ellipsometry, measurements of shear elastic constant of the monolayer and electron microscopy studies.
b) We are developing a general method of 2D crystallization using a lipid bearing Nickel which interacts strongly with the his-tag proteins.
c) We have analysed 2D protein crystals in situ using X-Ray diffraction.

The absorption of the protein at the lipid surface was monitored by ellipsometry measurements. The kinetics of absorption is about 200s to 500s. Ellipsometry is not sensitive to the lateral cristalline order so we designed an experiment for measuring the shear elastic constant of the monolayer. The lipid layer does not oppose a resistance to the flow but when proteins are added, a resistance appears slowly after ~1000 s and reaches a typical value of 1 to 3 mN/m This is correlated with the presence of crystals on electron microscopic grids. Cholera toxin B subunit (CTB) and Annexin V have been extensively studied and so is used as model proteins. The CTB interacts with a cell membrane receptor, a monosialoganglioside (GM1), Annexin V interacts with DOPS.
A His-tag transcription factor hupR has been successfully crystallized under a lipid-nickel monolayer and is tested with the techniques described above. A projection map at 20 Å is proposed.
We analysed the 2D protein crystals with the new high resolution grazing incidence diffractometer available at the ESRF. We have succeeded in measuring the diffraction pattern of 3 different 2D protein crystals up to a resolution of about 15 $Å^{-1}$. The intensity distribution along the rod scan has been measured. We have measured a coherence length for the crystalline order greater than 4 µm with the HupR 2D crystals. The irradiation damage is serious and leads to the disappearance of the signal after 50 to 100s.

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9-1

H. Berendsen.

No abstract available.

9-2

MOTIONS OF GROUPS OF ATOMS IN DNA STUDIED BY MOLECULAR DYNAMICS SIMULATION. D. Genest

The analysis of MD simulations of oligonucleotides reveals correlated motions of groups of atoms which can be considered as rigid sub-units. The analysis is performed as follows :

1 - Search for rigid sub-units : A rigid sub-unit is defined as a group of atoms exhibiting small interatomic distance fluctuations. Therefore the interatomic distance RMS's of the whole molecule are calculated and stored in a matrix. Then atoms with small RMS are regrouped. It is found that each nucleotide can reasonably be considered as composed of three sub-units : the base, the sugar ring and the backbone.

2 - Sub-units motions : For each sub-unit, the trajectories of the center of mass, of the Euler angles and of the constituting atoms are calculated. For these last ones, the coordinates are expressed in a referential bound to the sub-unit. Therefore the three kinds of trajectories correspond to the translation, the rotation and the internal deformation of the sub-units respectively.

3 - Correlation coefficients (equal time correlation): Using the canonical correlation analysis of groups of variates, correlation coefficients between the three types of motions of the different sub-units are calculated. This analysis shows that the deformation of the sub-units is not correlated to the translational or rotational motions.

4 - Conformational memory of DNA : Using the same mathematical approach as in 3, a canonical correlation functions study allows to determine how long DNA keeps the memory of its conformation. Translational motions of sub-units are long time correlated whereas rotational motions have correlation times on the order of 10 ps.

The same results have been obtained with two double stranded oligonucleotides (B-form) with different lengths and sequences.

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9-3

FOLD FAMILIES IN THE CATH DATABASE OF PROTEIN DOMAIN STRUCTURES AND IMPLICATIONS FOR ASSIGNING FUNCTION TO GENOME SEQUENCES. C.A. Orengo, A.D. Michie, S. Jones, D.T. Jones, M.B. Swindells, R.A. Laskowski, D, Milburn, J.M.Thornton.

The numbers of protein structures determined has risen exponentially over the last ten years with nearly 6000 crystallographic and NMR structures currently deposited in the Protein Databank (PDB). We have clustered all the well-resolved protein structures into families using both sequence and structure comparisons. Families are stored in a hierarchic database (CATH). CATH is an acronym for the various levels in the structural hierarchy. These are (C)lass, (A)rchitecture (describing the orientations of secondary structure elements), (T)opology (describing the connectivity between secondary structures or the fold of the protein) and (H)omologous superfamily. This latter level groups together proteins having significant sequence similarity or high structural and functional similarity indicating divergence from a common evolutionary ancestor.

In the September 1996 release of the CATH database, containing ~8000 domain structures, we identified ~650 homologous superfamilies (H), which collapsed to 506 unique fold families (T). Each homologous superfamily within CATH has been annotated by functional information obtained either from the literature, publicly available databases (e.g.SWISSPROT) or using in-house programs (XCITE, LIGPLOT, SAS). A method has been develped for automatically generating consensus templates (CORA, Orengo, 1997) for each homologous superfamily within CATH. Templates describe conserved features at different positions in the structure including the active site and ligand binding regions.

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9-4

WHAT CAN WE LEARN FROM MOLECULAR DYNAMICS SIMULATIONS OF RNA ? E. Westhof and P. Auffinger.

For several years, we have been trying to apply molecular dynamics simulations to large RNA systems. As a model system, we used the aspartic acid transfert RNA, which is already thoroughly described by crystallography in a free state and in a complexed state with its cognate synthetase. Simulations, done in vacuo or with empirical dielectric functions depending on the distance (r or 4r), gave trajectories in which the anticodon loop lost entirely its structure (in vacuo) or folded back either on itself or within the deep groove of the hairpin helix. Simulations were then performed in aqueous solution and in presence of counterions for neutralizing the solute, but with approximations on the computation of the long-range electrostatic forces via the introduction of cut-offs. Although the structures remained more stable, several artefacts were noted and the characteristic tertiary contacts were soon lost during the simulations. Finally, periodic boundary conditions with full computations of electrostatic interactions via the Ewald summation produced reproducible and stable trajectories during molecular dynamics simulations. The systematic use of multiple trajectories instead of a single long trajectory extended further the exploration of the conformational landscape. Simulations on the anticodon and on the full tRNA revealed the importance of C-H...O bonds within the solute and between solute and solvant. Besides, most of the hydration sites seen in crystallography were observed in the trajectories. Stereochemical rules for the orientation of the hydroxyl group were also uncovered.

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9-5

MOLECULAR MODELLING AND ELECTROSTATIC PROPERTIES OF THE PORE DOMAIN OF LIGAND-GATED RECEPTORS.
C.Adcock, G.R. Smith and M. S. P. Sansom.

The superfamily of neurotransmitter gated receptors consists of pentameric membrane proteins, which form ion channels in muscle and neuronal postsynaptic membranes. The nicotinic acetylcholine receptor (nAChR) forms cation selective channels and mediates excitatory synaptic transmission. Other receptors (eg. the glycine receptor, GlyR) initiate inhibitory synaptic transmission by forming anion selective channels. In this work we have concentrated on the transmembrane domain of GlyR and nAChR. Each subunit is thought to have four transmembrane segments, M1-M4. A large body of mutatgenesis and labelling data suggests that the M2 segment is α-helical and pore-lining. We have constructed models of bundles of M2 α-helices for several nAChR and GlyR using simulated annealing via restrained molecular dynamics. Restraints were generated from electron microscopy data and mutatgenesis data were used to determine orientation of the helices with respect to the pore. The electrostatic properties of the models have been assessed using Poisson-Boltzmann continuum electrostatics to estimate the electrostatic potential energy of a cation (E.P.E. (+)) as it passes along the pore. The results of these calculations have enabled experimentally observed selectivities to be predicted. Poisson-Boltzmann calculations have also been used to estimate pK_a values of rings of ionisable amino acids found at either mouth of the M2 helices. These residues are believed to have a key role in determining channel selectivity. The results have indicated that about half of the glutamate residues lining the extracellular mouth of the homomeric α7 nAChR pore may be protonated at neutral pH.

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9-6

From secondary structure prediction to fold recognition.

V. Di Francesco*, P.J. Munson* & J. Garnier+

Ab initio prediction of protein structure from the amino acid sequence alone is not yet possible. However useful approximations of the protein structure can be obtained if the amino acid sequence is homologous to that of a known structure. In order to detect the similarity of fold between sequences of less than 25% identity, we present a new approach based on sequences of secondary structures alone. A secondary structure prediction is performed on a sequence of amino acid residues and treated as a Markov chain of three letters: H for helix, E for beta strand and C for aperiodic structure. This predicted sequence is aligned to various hidden Markov models (HMM) of the actual secondary structure sequences of proteins having a specific topology. A protein topology recognition experiment is regarded as successful when the secondary structure sequence of a protein is ranked higher by the HMM of the correct topology than by HMMs of other topology families. For 24 proteins belonging to three structural classes, all-α, α–β and all–β, the success rate of fold recognition after crossvalidation was 63% with predicted secondary structures and 79% with observed ones. A similar rate of success was obtained on blind prediction experiments performed for the second Critical Assessment of Techniques for Protein Structure Prediction. This method can easily be used to screen entire genomes.

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9-7

COMPUTER SIMULATIONS OF PROTEIN-DNA INTERACTIONS.
Mats Eriksson and Lennart Nilsson.

We have simulated the DNA binding domains of the glucocorticoid receptor (GRDBD) and of the estrogen receptor (ERDBD), and try to establish the nature of the specific interactions between the molecules (DNA-protein and protein-protein). Several simulations of the free proteins in solution, as well as proteins bound to various DNA response elements have been made. Free energy perturbation calculations have been performed on the relative affinities of the wildtype GRDBD for different DNA response elements, yielding results in agreement with experiment, and some suggestions concerning the structural reasons for cooperativity, or lack thereof, in some complexes (Eriksson&Nilsson, 1995;Zilliacus et al., 1992). Structural and dynamic effects of point mutations in the P-box are found in the complex and in particular regions of the DNA binding domain of the protein.

Eriksson, M.A.L. & Nilsson, L. (1995) Structure, Thermodynamics and Cooperativity of the Glucocorticoid Receptor DNA-binding Domain in Complex with Different Response Elements, J. Mol. Biol, *253*, 453-472.

Zilliacus, J., Wright, A.P.H., Norinder, U., Gustafsson, J.-Å. & Carlstedt-Duke, J. (1992) Determinants for DNA Binding Site Recognition by the Glucocorticoid Receptor, J. Biol. Chem. *267*, 24941-24947.

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9-8

PROTONATION AND REDOX STATES OF THE QUINONES IN THE REACTION CENTER OF *RPS. VIRIDIS*.
B. Rabenstein, G. M. Ullmann, E. W. Knapp.

We calculated protonation patterns of the photosynthetic bacterial reaction center (bRC) of *Rps. viridis* for all protonation and redox states of the two quinones Q_A and Q_B, which may occur during electron and proton transfer processes. The conventional method of solving the Poisson Boltzmann equation on a grid with subsequent Monte Carlo titration was performed in conjunction with conformational changes. The latter was achieved by alternating energy minimization with the calculation of protonation pattern in an iterative manner until consistency of minimized structure and protonation pattern was reached. The relative energy of the quinone states in the different structures was also calculated by electrostatic methods, taking in account all protonations changing in the different quinone states by a complete Boltzmann-weighted sum.

The proton uptake of the bRC does not depend on the protonation state of Q_B but on the redox state. After electron transfer to Q_B the bRC already takes up a proton, which is later transferred to Q_B.

The conformational changes due to the energy minimization are all very small: The rmsd to the x-ray structure is about 0.1 Å in all states, the rmsd between minimized structures of different states is 0.01 to 0.02 Å. These small changes, however, are able to shift the redox potential of the quinones by several hundred mV. This shows clearly the great influence of conformational changes on the energetics of electron transfer and explains why earlier calculations neglecting conformational changes got the redox potential difference between Q_A and Q_B with wrong sign.

The calculated pK_a values of the first and second protonation of Q_B are consistent with experimental results. Q_B^{2-} is according to our computations in the bRC energetically even more unfavorable than in solution. Thus the transfer of the second electron does not happen until Q_B receives the first proton. The equilibrium of Q_BH and Q_B^- is inclined to the latter, so that the single protonated state can not be detected by IR spectroscopy. If the first protonation occurs much faster than the second electron transfer (this is corroborated by the fact that the proton enters the protein already after the first electron transfer), our results explain the pH dependence of the electron transfer rate and are consistent with recent kinetic studies.

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9-9

MODELING STUDIES ON ERYTHROCYTIC DYNAMICS: BETWEEN FACTS AND IDEAS. M. S. Almeida

The erythrocytic aging process is extensively characterized. Nevertheless there are still open questions. This study refers to the fundamental question of knowing how the erythron evolves towards a younger circulating population under conditions of accelerated erythrocyte aging. Thus it also concerns the limit of its response or the damage rate conducive to an impaired population.

Erythrocytes are normally exposed to various free radical species - including those released by activated phagocytes - known to cause cell damage (or aging). Moreover, circulating erythrocytes can be divided into age strata whose response is expected to be different. This work reports a cellular automata simulation approach used to follow the pattern of change of the erythrocytic population.

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9-10

THEORETICAL STUDY OF THE DARK ADAPTED STATE OF BACTERIORHODOPSIN J. Baudry, S. Crouzy, B. Roux, J.C Smith

Bacteriorhodopsin, the protein of the purple membrane of *halobacterium salinarium*, contains a chromophore (the retinal), whose conformational transitions are responsible for the function of the protein. After several minutes in the dark, the protein goes to an equilibrium state, called the dark adapted state. In this state, two isomeric forms of the retinal are found: in one, the retinal is all trans, and in the other, the double bonds C13=C14 and C15=N16 are cis. The population ratio (all-trans / (13,15 cis)) is 33%, wich gives a free energy difference of about 0.4 kcal/mol between the two conformers. In our work, we compute the free energy difference between these conformers of retinal, using molecular modelling techniques. Our ab initio quantum chemistry computations and our computation on *in vacuo* retinal show that the all-trans conformer is of lower free energy than the (13,15 cis) one by 1.5 to 2 kcal/mol. Molecular dynamics simulations of the retinal in the protein give the figure of about 0.2 kcal/mol for the free energy difference between the two conformers, of which the (13,15 cis) conformer is the stabler one. These results, which are in good agreement with the experiment, show that the nonbonded environment of the retinal in the protein is responsible for the lower free energy of the (13,15 cis) isomer.

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9-11

STRUCTURE ANALYSIS OF D- AND L- AMINO ACIDS HEXAPEPTIDES CALMODULIN MODULATORS AS DESIGNED BY SYNTHETIC COMBINATORIAL LIBRARY. NMR AND SYSTEMATIC CONFORMATIONAL SEARCH. V. Esteve[1], R. Tejero[1], D. Monleon[1], E. Perez-Paya[2], S.E. Blondell[3] and B. Celda[1]

Synthetic combinatorial library (SCL) methodology has become an important tool for the study of protein/ligand recognition that results in specific biological functions. SCL strategy has allowed to design D- and L- amino acids hexapeptides that bind to and selectively inhibit calmodulin. Such interactions between naturally ocurring L-amino acid proteins and synthetic all D-amino acid peptides open a new area in the stereospecificity of molecular recognition at the protein/protein level. As a part of a detailed structural study of these interactions between peptide and protein, the conformation of two chirality opposite hexapeptides is analyzed by NMR and systematic conformational search. A series of DQF-COSY, TOCSY and ROESY spectra have been obtained for both D- and L- amino acid hexapeptides. Simultaneous restrained simulated annealing (RSA) and systematic conformational search calculations have been applied to hexapeptide structure determination. A large part of identified NOE's does correspond to not contigous residues. In spite of their inherent flexibility, lower energy L-hexapeptide structures from RSA can be grouped in two well defined families, one of them having right-handed α-helix ϕ and ψ angles, which in part is supported by CD measurements. An α-helical conformation has been observed for natural ocurring oligopeptides that bind to calmodulin.

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9-12

GENETIC ALGORITHMS IN LOW RESOLUTION PROTEIN STRUCTURE FROM X-RAY SOLUTION SCATTERING

P. Chacón, F. Morán , J.F.Díaz, E. Pantos and J.M. Andreu

We present a new method for the characterization of the low resolution structure of proteins from solution Small Angle X-ray Scattering (SAXS) data. We focus this work on the inverse scattering problem which consists in deducing the possible structure(s) or shape(s) or structural changes of a macromolecule given its X-ray scattering profile to a given resolution. Contrary to the direct scattering problem, the inverse problem can not be solved analytically (1). The method presented here is used to fit experimental scattering profiles by applying a genetic algorithm (2) search in a given configurational space of mass distribution. The effectiveness of the procedure is demonstrated with synthetic objects, and by deriving 1.5 nm resolution models of known protein structures from their corresponding SAXS profiles. Performance results on simulated SAXS data derived from known structure coordinates are presented demonstrating the capability of the search procedure in finding models compatible with both shape and SAXS profiles. Preliminary results with actual experimental SAXS data validate the good performance and applicability of the method.

(1) Glatter O. & Kratky. (1982). Small Angle X-ray Scattering. Academic Press London

(2) Davis, L. (1991). Handbook of Genetics Algorithms Van Nostrad Reinhold, New York

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9-13

SIMULATION OF BIOLOGICAL GELS CONTRACTION BASED ON A MECHANOCELLULAR MODEL OF CELL - EXTRACELLULAR MATRIX INTERACTIONS. I. Ferrenq and P. Tracqui

We simulate the spatio-temporal behaviour of a mathematical model describing the mechanical interactions between cells and the surrounding extracellular matrix (ECM). The model takes into account cell migration as well as traction forces exerted by the cells, which are balanced by the passive mechanical response of the ECM. Furthermore, we consider that one boundary of the gel is free to move: this is indeed the situation encountered in several experimental situations dealing with gel compaction (microsphere compaction assays, gel compaction in petri dishes, contraction of free floating gels, ...). Using a finite differences approach, we solve in one dimension the system of partial differential equations describing the mechanocellular model and investigate its dynamic behaviour under different assumptions. First, we simulated the evolution of cells and ECM concentrations in time and space, and the associated ECM contraction, when the cell-ECM composite is modelled as a viscoelastic medium. Then, we analyse the modifications induced by considering that the ECM exhibit a visco-plastic behaviour. We show that the simulated contraction curves compare favourably to the experimental gel time-evolution thickness obtained in collagen gel compaction experiments. In addition, we investigate the influence of the ECM degradation on the kinetic of the ECM contraction. According to the model parameter values, different contraction profiles can be observed, with patterns composed of single or multiple contraction phase, possibly with alternating additional relaxation phase. These simulated contraction profiles qualitatively reproduce the experimental cell traction curves profile obtained with force measurements devices.

These two kinds of qualitative agreements suggest that the proposed mechanocellular model incorporates the main aspects of cell - ECM mechanical interactions. We more precisely discuss how such models can be valuable tools both for a quantitative estimation of these interactions and for a comprehensive view of traction cell mediated biological tissue organisation, as it occurs during wound contraction and remodelling.

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9-14

VAST, VECTOR ALIGNMENT SEARCH TOOL: AN ALGORITHM FOR COMPARING PROTEIN 3D STRUCTURES.. J-F. Gibrat*, T. Madej, J.L. Spouge and S.H. Bryant.

VAST is a method designed to find common 3D substructures in proteins. The algorithm is constituted of 2 stages.

The first stage uses a simplified representation of the polypeptide chain. Proteins are described exclusively in terms of their secondary structure elements (SSEs), α helices or β strands, each SSE being represented by a vector. For each common 3D substructure found between 2 proteins we calculate the probability of observing this similarity by chance only. This probability is a function of 3 factors i) the likelihood of generating a similar 3D substructure by chance, which is calculated using an empirical distribution of root mean square deviation (rmsd) for pairs of vectors randomly chosen in two different proteins, ii) the size of the proteins being compared, i.e., the number of SSEs in each protein and iii) the size of the database used. This probability, allowing the distinction between significant and chance occurrence similarities is the fundamental point of our method.

The second stage consists in the actual determination of an alignment for the Cas of the significant common substructures found in the first stage. During this stage an attempt is made to extend the alignment in the loop regions between the regular SSEs. A Monte Carlo procedure searches for the « best » alignment, i.e., the alignment that maximizes the Z-score, using empirical distributions of the rmsd calculated with sets of randomly chosen segments in proteins.

We have carried out a cross comparison of the whole PDB databank. The results have been included into the NCBI retrieval system Entrez, URL: http://www.ncbi.nlm.nih.gov/Entrez/

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9-15

MONTE CARLO SIMULATION OF NONSPECULAR X-RAY SCATTERING PROFILES FROM ORGANIC MULTILAYERS.

B.Gladyszewska, R.Koper and G.Gladyszewski[£].

We propose a simple model which allows to investigate the influence of different structural effects on the nonspecular X-ray scattering (NXRS) profiles from organic multilayers. In the frame work of the model the experimental ω-scan and ω-2θ measurements are mimicked for multilayers of dimyristoylphosphatidylcholine (DMPC) modified with a carotenoid pigment zeaxanthin. The model is split in two practically independent parts: i) Monte Carlo simulation of the multilayer structure taking into account interface imperfections or/and other desired structure effects; ii) Calculation of scattered X-rays from the simulated structure using the kinematical theory of scattering. This organization of the model - and therefore of the computer program based on it - allows to introduce any structural changes in a very simple way and to obtain a direct comparison between an image of the structure and the NXRS profiles. We studied the influence of uncorrelated, laterally correlated, and vertically correlated roughness on ω-scans and ω-2θ scans. The results show that the model is very useful to analyze the changes in the NXRS profiles. However, the fact that scattered intensity calculations are based on the kinematical theory of scattering makes it impossible to study the effects that have their origin in the multiple scattering, as Yoneda peaks and the near θ_c reflection. The simulations show that an interpretation of NXRS profiles cannot be performed for a single ω-2θ or ω-scan, but should be based on the analysis of I(ω,2θ) profiles collected over the whole (q_x,q_z) reciprocal plane.

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9-16

A BROWNIAN MOLECULAR DYNAMICS STUDY OF α-CYCLODEXTRIN – PHOSPHOLIPID ASSOCIATION. M. Göschl and S. Crouzy

Cyclodextrins (CD) are cyclic macromolecules formed by a closed ring of α(1,4)-linked glucose residues. The torus like shape of CD provides a central apolar cavity which enables the molecule to form inclusion complexes with a great number of *guest* substrates, ranging from nobel gas atoms to bulky polyaromatic compounds, e.g. drugs. However, CD are reported to exhibit a haemolytic activity that yet precludes its extensive parenteral use in pharmaceutical applications. It is generally believed that CD induced haemolysis occurs through the association of CD with membrane lipids, leading to a destabilization and rupture of the membrane structure. Our theoretical study uses molecular dynamics force field calculation to investigate the approach of α-CD towards homogeneous bilayer surfaces constituted of either phosphatidyl-choline (PC), -ethanolamine (PE), -serine (PS) or -inositol (PI) lipids. Two different simulation methods are used: i) Single 100 ps runs of Langevin dynamics using a CHARMM-type force field without repesentation of explicit solvent ii) 10^4 runs of rigid body Brownian dynamics (BD) of α-CD in an electrostatic potential that incorporates solvent and physiological ionic strength. In the first case, starting configurations were chosen as either the primary or secondary hydroxyl groups of α-CD facing the membrane at a distance of 40 Å. For BD-simulations, α-CD was randomly oriented prior to each run. The simulations show two remarkable features: i) Only PI and PS lipids are approached by α-CD while PE and PC do not exhibit any significant attraction. This point is in excellent agreement with observations made by NMR spectroscopy. ii) Attractive interaction strongly favours an orientation of α-CD with its primary hydroxyl side facing the membrane. Both observations can be explained in terms of the electrostatic dipolar interaction between α-CD and the lipid head groups.

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9-17

PROTON DELOCALIZATION IN PEPTIDE CRYSTALS AND IN COAL: MOLECULAR DYNAMICS SIMULATIONS. L. Henckes, R. Papoular, J.C. Smith

INS studies of N-methylacetamide and polyglycine crystals have been performed. The spectra have been intrepreted as showing a delocalization of the proton in the peptidic bond. Similar studies of different sorts of coals have also put in evidence nearly free protons.
In this contribution we present molecular dynamics simulations of a polyglycine crystal and of a simple model for coal. In the discussion of the results we reconsider the question of the delocalization of protons in both systems.

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9-18

A STUDY OF THE METHOXYL DISTRIBUTION IN PECTINS BASED ON NMR AND ENZYMIC DE-ESTERIFICATION. L. Catoire, R. Goldberg, C. Morvan and C. Hervé du Penhoat.
Pectins, which are a major constituent of the primary cell walls of plants, consist primarily of α-(1→4)-linked *D*-galacturonyl sugars occasionally interrupted by (1→2)-linked α-*L*-rhamnopyranosyl residues and about 70% of the galacturonan carboxyl groups are methylated. Besides their structural and functional roles in higher plants, these macromolecules are extensively used as gel formers and thickening agents in the food industry. *In vivo*, de-esterification is conducted by cell-wall enzymes (pectinmethylesterases or PME). In the case of PME of plant origin, the nature (random or blockwise) of this reaction has not been clearly demonstrated. This processus is of considerable interest as the methoxyl distribution strongly influences the rheological properties of pectic gels. We undertook the study of the action pattern of PME from *Vigna radiata* with various methods (NMR, viscometry, potentiometry, SEC etc.).
To overcome the unfavorable signal-to-noise ratio in NMR spectra of native pectins, a thermal depolymerization procedure was developed which reduces the weight-average molecular weight by a factor of ten without detectable modification in the primary structure. The chemical shifts of several signals in the ^{13}C spectrum are influenced by next-neighbour effects of both preceding and following residues and allow identification of triad structures such as UEU, EEE, UUU etc. (U and E stand for unesterified and esterified sugars). A Bernoullian distribution of triads was established as a function of DE and partial block distribution was demonstrated for a variety of pectins. Kinetic studies showed that substrate recognition by PME was not modified by the depolymerization routine and partial de-esterification of highly methoxylated samples with PME will reveal the nature of this reaction.

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9-19

CORRELATED MOTIONS IN LYSOZYME STUDIED BY MOLECULAR DYNAMICS SIMULATION. S.Héry, D. Genest, J.C Smith

Various theoretical and experimental methods have shown that atoms in proteins undergo liquid-like diffusive internal motions involving anharmonic, collisional local motions. At the same time, vibrational motions and conformational transitions correlated over long distances exist.

To further investigate collective internal motions in lysozyme, we analyse a 1 *ns* molecular dynamics simulation of crystalline orthorhombic hen egg-white lysozyme in terms of rigid-body displacements. From the root-mean-square distance fluctuation matrix (1) or the canonical cross-correlation matrix (2), rigid-body groups can be extracted and analysed.

We report here the results obtained by these two methods and the influence of these rigid-body motions on the X-ray diffuse scattering pattern.

(1) S. Héry, D. Genest, J.C. Smith *Physica B* (1997) in press.
(2) F. Briki, D.Genest (1994) *Biophysical Chemistry* **52**, 35-43.

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9-20

MONTE CARLO METHODS FOR EFFICIENT SAMPLING OF BIOMOLECULAR SYSTEMS. D. Hoffmann

Computational methods in biomolecular research offer a detailed picture of the dynamics at the molecular level. Traditionally, the method of choice has been Molecular Dynamics (MD). Here it is shown that Monte Carlo (MC) sampling using suitable move sets can be a valuable computational tool, too. This is mainly because MC sampling allows for larger conformational changes, and hence a better sampling of conformational space. To demonstrate the capabilities of MC sampling, mixtures of cartesian and dihedral moves are applied to several biopolymer systems. The molecular models are the same as in conventional MD, i.e. molecules are modelled in atomic detail. MC trajectories are used to calculate thermodynamic averages, as well as to obtain insight into dynamic phenomena (folding). Disadvantages of the presented MC method to date are the necessity of a move set optimization in order to achieve good sampling efficiency, and the qualitative representation of solvent.

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9-21

MOLECULAR SIMULATION OF THE EQUILIBRIUM BETWEEN TWO CAPPING BOXES IN THE A-HELIX OF ANNEXIN I DOMAIN 2. T. Huynh, J. C. Smith, J.M. Neumann and A. Sanson.

The isolated annexin I domain 2 is essentially unfolded in pure aqueous solution. Detailed NMR analysis of the unfolded state of the annexin I domain 2 shows the presence of both native and non-native residual local structures. Our background hypothesis is that the non-native local structures, as well as the native one's, actively control, structurally and kinetically, part of the folding pathways of the protein. The A-helix of domain 2 contains such a non-native structure, namely a capping box in the middle of the helix in addition to the native one at the N-terminus. These canonic capping boxes TxxQ and DxxE are underlined in the following sequence:

L K T P A Q F D A D E L R A A M K G

Interestingly, the two capping boxes interact with each other as revealed by NMR study of the above fragment. The basis of this interaction was analysed by point mutation and NMR (see poster of R. Guerois *et al*). To complete this study we have undertaken the molecular simulation of the fragment and of 8 mutants. The aim of this work was to calculate from the simulation trajectory the NMR parameters (NOEs, JαN coupling constants) for comparison with the average experimental values. The CHARMM program with explicit solvent molecules was used. The role of the Asp8 residue in the helix breaking was specially emphasized. The results of the molecular dynamics simulation were found in reasonable agreement with NMR data. For example, the original sequence was found to unfold at D8 residue but the D8A mutant remains stable as it was experimentally observed. These simulations were also aimed at understanding the role of water molecules in the unfolding of the A-helix. Comparison between the full set of NMR and simulation data is expected to improve the conditions of a realistic simulation of protein unfolding.

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9-22

A MONTE CARLO METHOD TO SIMULATE THE PROTEIN FOLDING PROCESS. Sartori, F., Hoffmann, D., Knapp, E.W

A new Monte Carlo (MC) method is presented, which allows to simulate the folding process of small oligopeptides in a realistic manner. An all atom, off-lattice protein model with rigid bond lengths, bond angles and amide planes is used. The only degrees of freedom of the polypeptide backbone are the (phi,psi)-torsion angles. An elementary MC move is performed by co-operative rotations in a small window of consecutive amide planes, leaving the polypeptide conformation outside of the window invariant. These window MC moves generate local conformational changes. Large conformational changes of a polypeptide evolve gradually in time, by applying these MC moves randomly many times.

To account for lack of flexibility in the employed protein model a potential of mean force is used for the (phi,psi)-torsion angles. It is derived from molecular dynamics (MD) simulations of a flexible dipeptide using a conventional MD energy function. To avoid exaggeration of hydrogen bonding strengths for rigid polypeptide models the electrostatic interactions involving hydrogen atoms are scaled down at short distances as compared to MD energy functions. With these adjustments of the energy function the rigid polypeptide model exhibits the same equilibrium distributions as does a fully flexible model with MD simulation.

The MC method is applied to a model peptide of 26 residues which represent the central part of the helix-turn-helix motive of ROP (Hoffmann, Knapp, 1996). Starting from a stretched beta-strand alpha-helical structure forms quickly. It follows a hydrophobic collapse where the turn appears but is displaced. Then in a slow process of self-repetation the turn moves to its native-like position. The final conformation of the model polypeptide agrees with the ROP structure within the uncertainties of the energy function used.

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References.
Protein dynamics with off-lattice Monte Carlo moves, Hoffmann, D., Knapp, E.W. Phys. Rev. E53 (1996) 4221-4224

9-23

PLASMA GLUCOSE LEVEL MODIFIES THE GLUCOSE METABOLISM OF BRAIN. A PET STUDY. Márián[#], T., Fekete[*], I., Balkay[#], L. and Trón[#], L.

This study evaluates the effects of hyperglycaemia and hypoglycaemia on fluorodeoxyglucose (FDG) uptake and glucose metabolism in the brain.
Methods: Dynamic PET studies with FDG were carried out using rabbit animal model. We applied glucose loading and insulin treatment (i.v.) to manipulate the plasma glucose concentration (in the range from 0.3 mM to 29 mM) of the rabbits. Serial arterial blood sampling was performed in all cases to allow kinetic analyses of FDG acummulation.
Results: Brain metabolism was quantified by the standardized uptake value (SUV) of FDG. The SUV values in hyperglycaemic animals (0.83 ± 0.19) were lower than that of the control groups (1.37 ± 0.46, $p=0.05$), while hypoglycaemia increased to 5.21 ± 2.5.
Quantitative data were obtained by using the three-compartment-model of FDG accumulation. Kinetic constant k_1, k_2, k_3 and k_4, and the cerebral glucose metabolic rate (GMR) were determined for the rabbit brain. The rate of FDG metabolism in the brain decreased by elevation of plasma glucose levels. Hyperglycaemia decreased the rate of FDG uptake (to 60% of control), while extreme hypoglycaemia (0.3-2 mM plasma glucose) increased 5-10 times the FDG metabolism of brain relative to the control. The kinetic constant of FDG phosphorylation (k_3) shows a close correlation ($p=0.05$) with plasma glucose level, as well as the kinetic constant (K_1) of FDG transport. The level of hypoglycaemic GMR was higher (8.15 ± 2.3) than that in hypoglycaemic brain.
Conclusion: The plasma glucose concentration has a significant effect on the glucose metabolism of the brain.
(This study was supported by OTKA grants T 16149 and T 16150.)

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9-24

A COMPARISON OF THE TIP3P, SPC AND SPC/E WATER MODELS. A MOLECULAR DYNAMICS STUDY OF TRYPTOPHAN IN WATER. P. Mark and L. Nilsson

Molecular dynamics simulations of different water models (TIP3P, SPC and SPC/E) were performed using the CHARMM program. The effects of different cutoff distances and methods were studied. The self-diffusion coefficient, D and radial distribution functions g_{OO}, g_{OH} and g_{HH} were determined and compared to experimental data. Molecular dynamics simulations of a single tryptophan molecule using different water models were carried out and reorientation times were compared to experimental values.

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9-25

MINIMAL MODEL FOR CALCIUM SIGNAL IN CYTOSOLIC, NUCLEAR AND RETICULUM ENDOPLASMIC POOLS OF EPITHELIAL CELLS. P. Seghieri, PM. Martin and C. Penel

Single cell analysis by confocal microscopy of calcium signal (Fluo3 probe) in cytosol and nucleus following Epidermal Growth Factor simulation in A431 cells gives rise to different signals which can be grouped in three classes ; a spike and a return to the basal level (signal A), a spike and a decrease to a plateau (signal B) or a slowly increase to a plateau (signal C). When a steady state is reached oscillations occurs. A minimal model is proposed for the interpretation of these results where the transfer of calcium ions between the three main compartments : cytosol, nucleus and endoplasmic reticulum (ER) is described by transition probabilities (Markow process). Plasma ROC channels and nuclear pore permeabilities are considered to be potential but not calcium dependent while the IP3 receptor permeability is described by a bell shape calcium function, plasma and ER CaATPase are described by Hill functions. The kinetic properties of these entities results from the fitting of previously published experimental data. A software written in C runs on a PC under Windows environment to simulate the evolution of calcium in the ER, a useful predictive aspect of the model since this compartment is not yet accessible experimentally without drastic experimental conditions. Computer simulations show that signal A can result from the activation of IP3 receptors while signal C would result from the activation of the ROC channels ; signal B appears as the additive contribution of both channels, while oscillations are compatible with a « calcium induced calcium release » mechanism. Simulations show that the calcium dynamic in the ER is generally a mirror of cytosolic and nuclear calcium but that a simple way to produce similar calcium elevation in these three compartments is to activate plasma ROC channels.

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9-26

STRUCTURAL AND DYNAMICAL MODELLING OF THE PRO-OCYTOCIN-NEUROPHYSIN PRECURSOR

B.Velikson, P.Cohen, J.P.Rose, B-C Wang, J.C Smith

We want to obtain a 3-dimensional structure for the hormonal precursor: Pro-Ocytocin-Neurophysin. Apart from the sequence, the experimental information avaliable is: (1) a 3.0Å-resolution X-ray crystallographic structure of *bovine neurophysin II*; (2) a crystal structure for an ocytocin–bovine neurophysin II complex; (3) some NMR studies of soluted fragments of the precursor. The precursor is known to be cleaved at the Arg12-Ala13 location. According to the statistics conducted on 265 dibasic aminoacids contained in 66 hormonal precursors, the preferred secondary structure sequence surrounding the cleavage site is α-helix–β-turn–α-helix. Combining vacuum MD simulations at constant temperature with simulated annealing and energy minimization, we obtained several candidate structures, in all of which: (1) the C-terminal of the precursor sits in a pocket formed by the neurophysin part; (2) res. 1-22 form a loop such that there is always a β-turn-like structure near Arg12, and Arg12 is more exposed that the surrounding residues; (3) the overall structure of the neurophysin I part is the same as in the crystal neurophysin II, but one can see a greater hinge-like opening between the 2 domains of neurophysin in the presence of the ocytocin part. A simulation of neurophysin II under the same conditions does not result in such effect, which suggests that this result is not an artefact due to the absence of solvent.

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9-27

REALISM OF THE PROTEIN SECONDARY STRUCTURE PREDICTION USING THE MULTIPLE SEQUENCE ALIGNEMENTS. K.Zimmermann.

Current prediction methods yield fuzzy results (in terms of individual residue state propensities -helix, beta,coil)). It is then necessary to interpret these propensities in terms of a realistic secondary structure[1]. The overall prediction qality can be improved by using a consensus prediction of multiple sequence alignements. However, the problem to assign the individual residue states is much more complicated: either one find realistic predictions for each of aligned sequences (but usually no consensus), or one find easily a consensus, usually not realistic.

We have generalized our preceding regularization method[1] to solve this problem.The new method finds the best realistic consensus secondary structure compatible with the predicted propensities At the input it reads the multiple sequence alignement (e.g. in CLUSTAL format) and the propensities file. At the output it yields the same alignement of the predicted secondary structures. .The method is based on the dynamic programming optimal path algorithm. It is quite objective (no heuristics) and the results are globally optimal.

Reference 1: K.Zimmermann: When awaiting 'Bio'Champollion: dynamic programming regularization of the protein secondary structure predictions, *Protein Engineering* 7, 1197-1202 (1994)

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10-1

LIPOSOME SIZE DETERMINATION USING A MICROTRACK UPA.

Barnadas R., Campillo M., Sabés M.

This work describes the use of the Microtrack UPA (Leeds & Northrup) as a tool for the liposome diameter determination. Using this device, the intensity/time pairs obtained from the reflected laser beam after interacting with the sample, are transformed through the Fourier space to intensity/frequence values. The size distribution is calculed from the Doppler shift, as consequence of the particle movement (diameter). The liposome suspensions were obtained from Prolipo S (Lucas Meyer), or from purified egg phosphatidylcholine and phosphatidyl acid (Sigma). Extrusions throught policarbonate membranes (800, 400, 200 and 100 nm) ,microfluidification or sonication were made when necessary. Two parameters of the Microtrack UPA were evaluated: the loading index, a measure of the signal level that the detector receives; and the run time, the time during which the device acumulates information from the sample. The useful loading index range was found to be dependent on the particles diameter, but showing a common minimum value (0.07 arbitrary units). This result disagrees with the manufacturer, who found an optimum and independent interval range form 0.1 to 1. When standard glass particles were used, the results concorded with the Firm. The effect of the run time was statistically evaluated studying the liposomes samples during 1, 3, 7.5 and 15 minutes. A critical value of 7.5 was determined in order to obtain reproducible lectures for all the liposomes. Finally, the entraped volumes of REVs extruded through different policarbonate membranes, and SUVs were calculated from the standard particle size distribution and compared with experimental values obtained using $Na_2Cr_2O_7$ as a marker of the internal volume. A good correlation was found from SUVs to 250 nm particle size. The divergence of the results difered with the vesicle size.

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10-2

FREE RADICALS FROM POLYCRYSTALLINE NUCLEOTIDES AFTER HEAVY ION BOMBARDMENT AND X-IRRADIATION

B.Dusemund and J. Hüttermann.

An early observable damage after irradiation, which causes cell death or cancer, is formation of free radicals inside the cell core and DNA. Electron Paramagnetic Resonance (EPR) is capable of detecting various types of radical structures in single DNA nucleotides subunits and in mixtures of nucleotides. In this way information is obtained about the relative sensitivity of parts of DNA with respect to free radical formation. These experiments were performed using both x-rays and heavy ion radiation in order to probr the dependence of mechanisms of radical formation from radiation quality. The nucleotides were freeze-dried in H_2O and D_2O, so that a H/D exchange can happen. The samples were irradiated at 77 K (ca. 100 K for heavy ions) and subsequently annealed to room temperature in order to induce transitions from primary to secondary radical species. The EPR spectra were analyzed by deconvolution algorithms in order to obtain elememtary radical patterns.

Single nucleotides were analyzed first and a radical pattern balance with temperature was established. The change in balance for nucleotide mixtures from dimers, trimers and from the tetramer containing all four nucleotides was probed using the elementary patterns. The most sensitive part of the DNA was found to be pyrimidine nucleotide TMP followed by another pyrimidine DCMP. In all mixtures containing TMP the radicals of this nucleotide are dominating.

This work was supported by a grant from the GSI (HOHÜB), Darmstadt, Germany.

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10-3

NONLINEAR BEHAVIOR OF POWER DENSITY AND EXPOSURE TIME OF ARGON LASER ON OCULAR TISSUES .

E.M. El-Sayed, M.S Talaat and E.F Salem.

The behavior of ocular tissues under the effect of 488 nm argon laser was investigated.Two constant doses of $7.2x10^{-3}$ and 260 x 10-3 W.s /mm^2, were used by varying both the laser power density p and exposure time t in different ways . The intraocular temperature distribution and electroretinogram (ERG) were examined under such laser conditions. Consequently , the thermal conductivity of cornea ,lens and retina were calculated and their values were found to be 0.5 +0.007, 0.364+ 0.003 and 0.073+ 0.005 W/m^0C respectively. The obtained results indicated that intraocular temperature distribution and ERG records were strongly temperature dependent and they mainly affected by the laser total dose . However , when the dose was kept constant , the number of laser shots, the exposure time , as well as the power density were found to be important factors for determining the magnitude of ocular temperature rise and ERG. The obtained results indicate the nonlinear behavior effects of argon laser on the intraocular temperature rise and ERG records .

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10-4

A METHOD TO MEASURE TRACE CONCENTRATIONS OF HYDROGEN (H_2) DISSOLVED IN URINE

M.A. Esteso, R. Rodríguez-Raposo and J.J. Podestá

Measurements of breath H_2 excretion is a useful non invasive method for detecting carbohydrate malabsorption. Gas chromatography and electrochemical methods using a polarographic type cell has been the most frequently techniques used to quantitatively analyze H_2 in end alveolar excretion after the ingestion of a non-absorbable disaccharide as lactulose.

There are, however, multiple references in the literature indicating that the breath H_2 technique has various limitations about both the technique itself and the protocol to obtain the samples (due to wide individual differences), that make difficult to quantify accurately the malabsorption of fermentable material.

Nevertheless, since this H_2 is present dissolved in urine it could be easily determined there if an accurate method would be available.

In the present communication we report a very simple and cheap method to measure this biological H_2 dissolved in urine. The method uses a novel electrochemical potentiometric sensor which is based on a fuel-cell constituted by a solid polymer electrolyte (SPE) ionomeric membrane coated with platinum. The sensitivity of such a sensor permits to determine trace concentrations of this biological H_2.

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10-5

DAMAGES PRODUCED IN VISCERAL SMOOTH MUSCLE CELLS RADIATED BY HIGH ENERGY PHOTONS. Ferreira,A.T.; França,J.P.; Santos,S.C.;Oshiro, M.E.M.; Segreto, R.A.; Segreto, H.R.C.; Haapalainen,E.F.

Purpose: To evaluate the effects of gamma radiation on: a) basal intracelular calcium and sodium; b) functional cellular alterations through the response to specific agonists: Angiotensin II (AII) and Acetylcholine (Ach); c) alterations on lipidic matrix through the changes in the plasmatic membrane permeability induced by the formation of gramicidin channels/pores; d) quantity of malondialdeid (MAD) produced by membrane phospholipids peroxidation; e) growth curve and survival of the cell cultures and f) morphologicals changes of the cells when exposed to radiation.**Methods and Results**:culture of smooth muscle cells from guinea-pig longitudinal layer were irradiated with high energy photons, around 1.25 Mev, with a Cobalto-60 source, and dosis betwen 250 to 5000 cGy were used and compaired to control cells (non irradiated). The free radicals were indirectly determined by photometric method, by measuring MDA whose concentration was related to the number of cells and indicated one constant ratio superior to 100% for the maxima dosis of gamma radiation. We observed an increase in number of inviable cells for higher dosis of radiation using the exclusion of the dye Triplan blue and photometric method with ethideo bromide. In the evaluation of the growth curve and mortality of irradiated cells they are statiscally different from control cells. The cytosolic calcium and sodium were measured by fluorimetric method using fura-2 and SBFI as fluorofore, respectively and are increased in the radiated cells. It was observed morphologicals changes in endoplasmatic reticulum, disappearing with high dosis, but the mitochondria was structuraly intact in all absortion dosis of radiation. The increase of cytosolic Ca and Na induced by AII and Ach are reduced in a dosis of radiation dependent manner. **Conclusion**, there are morphological, biochemical and functional changes under high energy photons radiation of SMC. **Financial support: CNPq**

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10-6

^{31}P NMR FOLLOW-UP OF DIFFERENTIATION INDUCED BY HUMAN RECOMBINANT BMP2 ON MOUSE FIBROBLASTS.

J. M. García-Cantalejo, J. Zurdo & J.L. López-Lacomba[1]

The bone morphogenetic proteins (BMPs) are involved in cellular differentiation and cell migration during embryogenesis. They also play a fundamental role in the healing of bone damage, being able to induce *in vivo* differentiation of mesenchimal cells into new bone throught a condrocyte step. The biological activity of these proteins can be tested *in vitro* with biochemical techniques, where it has been reported effects on the Phosphatase alkaline activity, collagen and osteocalcin syntesis. Unfortunately, usual activity essays cannot be performed *in vivo*, since only the sacrifice of the experimental animal and/or the use of histological staining are currently employed to confirm cell differentiation. To solve this problem, the use of a non-destructive, non invasive technique like NMR will be a great advantage if it showed be able in provide relevant information about the success of the differentiation process. The objective of our study has been to find a correlation between the biochemical changes induced by BMP proteins during differentiation, and the levels of different metabolites detected by ^{31}P NMR. To this aim, human recombinant homodimeric BMP2 has been obtained and employed to test its biological activity on mouse fibroblastic cell line C3H10T1/2, where it has been reported the ability of BMP2 to induce osteodifferentiation. Base line ^{31}P spectra were recorded perfunding HHBSS (Hepes Hanks Buffered Salt Solution without Pi). After 4 days incubation with 30μg/ml of rh-BMP2 cells showed a significant variation on PE and NTP levels, that can be correlated with the altered metabolic activities previously reported. These results shows the NMR spectroscopy as a useful tool to determine direcly the activity of osteoinductive factors on tissue and cell line differenciation processes.

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10-7

PROGNOSTIC VALUE OF THE 0°C ISOTHERMIC PERIOD IN CRYOLESION SIZE AND GEOMETRY. A. Hoekstra, C.D.J. de Langen, P.G.J. Nikkels, B.J. Korteling, K.J. Bel and H.J.G.M. Crijns.

Radiofrequency is the usual energy source for clinical ablation of cardiac arrhythmias. Surgical experience has shown that cryoablation is also effective for ablating arrhythmogenic substrates. A new steerable 8.5F bipolar catheter fitted with a feedback thermocouple was tested in 7 anesthetized pigs (30 kg) guided by the electrocardiogram in order to modify the AV nodal and His-Purkinje system conductive properties. Thermal energy was delivered by a N_2O tank (> 650 psi) via a cardiac cryo unit (Spembley, Hampshire, UK) into the catheter wherein gas expands resulting in a tip temperature as low as -70 ± 2°C within 10 seconds. Cryoablation under fluoroscopic and electrocardiographic guidance was applied at multiple sites in both ventricles for 30, 60 or 120 seconds. After a follow-up period of 6 weeks, the AV nodal conduction in the pig hearts had been affected persistently. Moreover, a significant QRS duration increase had been achieved as a result of the procedures (32.2 ± 14.3 %; $t = 4.37$, $p < 0.05$, paired t-test). The ablative lesions found were well demarcated with small margins of hypertrophy of myocardial cells. With respect to lesion volume variability (8-450 mm^3) and geometry, a relationship between isothermic period and cryolesion volume was found. Results of an in vitro model confirmed this relationship. Therefore, an isothermic period probably can predict the lesion size.

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10-8

RELEASE OF UNALTERED BASES AFTER HEAVY ION BOMBARDMENT AND X-IRRADIATION OF DNA COMPONENTS. A.-K. Hoffmann and J. Hüttermann.

Important effects of ionizing radiation on living organism are attributed to chemical modifications induced within DNA. An early effect of radiation is the formation of radical species which are the precursors of these modifications like modified bases and strand-breaks. Direct radiation action involves deposition of energy in the target molecule itself. Free radicals induced upon heavy ion bombardment and X-irradiation by direct radiation action are mainly products of the ionization pathway due to the reaction with electrons generated through interactions of the primary radiation with the target.

DNA and its components irradiated as solid samples were used in order to study explicitly the direct radiation effect. As first product, the release of unaltered bases after heavy ion bombardment and X-irradiation was investigated using HPLC and NMR. After irradiation of pyrimidine nucleotides as models for nucleic acids the main diamagnetic products were the free bases thymine or cytosine, respectively. The increase in the yield of free bases was linear with a dose up to 200 kGy for heavy ion bombardment or 120 kGy for X-irradiation. The estimation of the radiation chemical yields for the release of the free bases revealed G values in the order of magnitude 10^{-7} mol J^{-1}. The heavy ion bombardment effected a lower base release than X-irradiation. The formation of free bases indicated that their radical precursors in the solid state are centered within the sugar moiety, probably connected with a strand-break in DNA.

This work was supported by a grant from the GSI (HOHÜB), Darmstadt, Germany.

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10-9

NUCLEAR MAGNETIC RELAXATION IN VIVO STUDY - METHOD FOR DETERMINATION OF DYNAMIC AND STRUCTURE PARAMETERS OF MEDIA / NEAR CELL WATER MOLECULES AND SUBSTRATE / BIOMASS CONCENTRATION.

R. S.-H. KASHAEV

On the base of our earlier constructed pulse Nuclear Magnetic Resonance-relaxometer an NMR-analyser for in vivo measurements in protons of near cell and media water in the process of microbial cultivation was designed. Owing to large sample probehead (30 mm) and precise temperature regulation $\pm 0.1^0$ C in the range 0-45^0 C the noninvasive cultivation could be done directly in the probehead of the NMR-analyser. The studies were devoted to the important problem - search of methods and microbial clones able to biodegrade highsul-phurous oils and oil pollutions. NMR-investigations made in soil bacteria ,cultivated for several days on the sulfur absent media with sulfurous oil, black mineral oil and dibenzothiophen as a source of sulfur, showed, that this method of control and NMR-analyser - are a good opportunity for operative, noninvasive in vivo control of biomass and substrate fractions concentration. Besides, the parameters of water molecules structure and dynamics could be determined through the set of NMR-parameters T_{2aw}, T_{2bw} and P_{bw}, corresponding to the water molecular protons in cultural media and in hydrate shells surrounding bacteria cells. It was found that this hydrate shells appear with bacteria growth, demonstrating periodicity. Values of T_{2bw} reach its minima at the maxima of bacteria concentration and vital activity, indicating the hydrate most ordered structure with the maxima of water molecules ordered population expressed by equation $P_{or} = 0.296\ T_{2bw}^{-1} - 0.078$. Hydrate shells proton population P_{bw} reflects changes in cells concentration and is proportional to optical density D and bacteria concentration through equation $P_{bw} = 0.195\ D + 0.051$.

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10-10

SPONTANEOUS FLUCTUATIONS OF THE RESTING POTENTIAL IN *NITELLOPSIS OBTUSA*.

B.Gladyszewska and R.Koper.

Many physical objects generate spontaneous fluctuations. It was found that the cell membranes also generate cellular potential fluctuations because of ion penetration through the membranes. In this work, we present some statistical properties of these fluctuations measured for *Nitellopsis obtusa*. Cells of *Nitellopsis obtusa* were placed in a grounded metal box. Both platinum and thin capillary glass liquid electrodes were used. The signal was amplified using a broad-band preamplifier of an input resistance 100 MΩ. The mean resting potential was measured and controlled with an oscillograph. An amplitude analyzer was used to measure the distribution of momentary amplitudes and a resonans amplifier to measure spectral density functions. A correlator allowed to study the autocorrelation functions. In all studied cells the distribution of fluctuation amplitudes had a Gaussian character. The autocorrelation functions revealed a very complex character and were difficult to interpret. Neither an exponential function nor a sum of exponential functions resulted in a sufficiently good fit. This fact draw the author's attention to a more readable function: the spectral density function $W(f)$. In a frequency range 10Hz-100Hz $W(f)=W_0$ was found. For frequencies higher than 1kHz $W(f) \propto 1/f$. When trying to fit a whole spectrum, an interesting approximation was obtained by the use of the function $W(f)=W_0/(1+a \cdot f)$. The results were reproducible only in the range of several minutes. It is obvious that natural processes in the cell may change some spectral parameters. Indeed, as follows from our measurements, the parameter W_0 changes. However, the general character of the spectrum (and, therefore, the *"a"* parameter) remains unchanged.

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10-11

INDUCED MORPHOLOGICAL CHANGES IN SYNTHETIC GIANT VESICLES: GROWTH, FUSION, UNDULATION, EXCRETION, WOUNDING AND HEALING.

F.M. Menger, S.J. Lee

Giant unilamellar vesicles, prepared by hydrating a synthetic lipid, are visible under phase-contrast microscopy. Additives injected into or onto the vesicles induce various morphological changes that have been recorded photographically. For example, KI/I_2 creates a large, slowly-healing hole in the vesicle surface to form a solvent-filled "nanocup". Sodium cholate also injures the vesicle surface, but the defect is smaller and heals rapidly. Sodium acetate induces vesicle fusion, a process explainable by the Svetina-Zeks mechanism. Polyvinyl alcohol causes filament-connected vesicles (but not isolated vesicles) to fuse. This observation leads to the speculation that many fusion experiments with submicroscopic vesicles might also reflect, unknowingly, the presence of intervesicular filaments. Severe osmotic stress, as provided by 0.1 M NaBr, forces the vesicles to undulation vigorously. Finally, injection of a fluorescent dye into the vesicles allows, via fluorescence microscopy, the detection of outward diffusion by the dye. Giant vesicles provide a particularly valuable membrane model because, unlike submicroscopic vesicles on which the bulk of bilayer research has they far been focused, the results are not affected by unnatural membrane curvature. Moreover, morphological changes can be monitored as a function of membrane composition and experimental conditions without relying on indirect spectroscopic methods.

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10-12

DIFFUSION OF CRYOPROTECTIVE AGENTS IN INTACT HUMAN ARTICULAR CARTILAGE. A NMR STUDY.

López-Lacomba, J.L.[&]; Carsi, B.[*]; Sanz-Casado, J.V.[A]; López-Durán, L.[*]; Marco, F.

A complete penetration of the cryoprotective agent in the human articular cartilage sample is basic for a successful cryopreservation. However the actual freezing protocols do not take into account any real experimental data about incorporation of the agents and are based on empirical knowledge, being a standard procedure to include the cartilage piece 30-45 min in 15% DMSO. To asses the penetration rates of different cryoprotective agents, fresh samples of human intact articular cartilage were retrieved from multiorganics donors. In order to preserve the tissue structure we chose a non destructive technique as NMR, using a Bruker 4.7T magnet. DMSO penetration rate was evaluated by analysis of the changes in image intensity relating it to the exposure time of the tissue to the agent. In this way we obtained a T_{50} value of 38 min. at room temperature. This data related unfavourably for the one for deuterated saline which is about 1.8 min. A possible reason would be a reduced self-diffusion coeficient (D) as compared to water. Those values have also been determined by NMR diffusion experiments finding a difference between both data around one order of magnitude ($1.3\ 10^{-7}$ vs $4.3\ 10^{-8}\ m^2/sec$). We also observed significant differences between D values for free DMSO in solution and that obtained inside the tissue ($2.1\ 10^{-8}$) reflecting the restriction imposed by the extracellular matrix. Similar experiments are being performed with glycerol, other common cryoprotective agent, finding a T_{50} value of 8.5 min. The results here obtained points to the standards cryopreservation protocol as a possible source of the *in vivo* heteroinjerts of articular cartilage failure (almost 50%), and suggests that alternative protocols that ensure the cells cryoprotection will improve clinical results.

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10-13

THE EFFECT OF BETA RAYS FROM Sr^{90} EYE APPLICATOR ON RABBITS' LENS LIPIDS. S.S. Mahmoud[1], F. M. Elrefaei[1] and F.M. Ali[2]
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This study aimed to throw light on the molecular basis by the complciations occure following exposure of NewZealand white rabbits to β-rays from Sr^{90} eye applicator in the range of therapeutic doses. This study included sixty rabbits classified into four groups namely A,B,C and D of fifteen rabbits each, five of them served as control. The right eyes of the rabbits received 10,20,40 and 60 Gy respectively in a weekly sitting of 10 Gy/week. Lipids of rabbits'lenses for both right (treated) and left (untreated) eyes and their corresponding control lenses of the used rabbits were studied using Uv-spectrophotometry ; quantitative determination of cholesterol and phospholipids in addition to examination of lipid classes via thin layer chromatogrphy. The results showed the changes in water content were not dose dependent. The typical absorption spectra of lipids extracted from lenses showed the formation of lipid peroxidation products (either as 1 ry and/or 2 nd products) in the treated eyes while those of untreated ones showed that same characteristics as the normal lenses for the administered doses. The results obtained from TLC indicated no change in the neutral lipid classes separated from lenses. We can conclude from this study that the exposure of the eye to beta particles led to damage of lens lipid components and the formed peroxidation products are associated with changes in the fluidity of the lenses.

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10-14

NUTRIENT SECRETAGOGUES INCREASE DIADENOSINE POLYPHOSPHATES CONTENT IN PANCREATIC ISLETS. F. Martín, C. Ripoll, J. Pastor, M.T. Miras and B. Soria.

Diadenosine polyphosphates (Ap_3A and Ap_4A) at concentrations found in glucose-stimulated cells are effective inhibitors of ATP-regulated K^+ channels. To investigate the role of DPs on insulin release, we have studied the various pathways for their synthesis in pancreatic islets. Methods: Islets from adult Swiss mice were isolated by collagenase digestion and incubated at 37° C in different media and at different times. After incubation, batches were sonicated in 50% water/ethanol and centrifuged for 30 min at 65,000 rpm and 4° C. Proteins in the supernatant were precipited in acetone. The final suspension was lyophilized and analyzed by HPLC. Results: 1°) Ap_4A and Ap_3A show a glucose-dependent cytosolic increase (half maximal concentrations: 7.8 and 7.4 mM respectively). 2°) Ap_4A and Ap_3A show a time-dependent cytosolic increase (half time: 152 and 167 s respectively). 3°) 10 mM Leucine and 10 mM ketoisocaproate induce a cytosolic Ap_4A (66- and 80- respectively) and Ap_3A (44- and 70- respectively) fold increase. 4°) 22 mM glucose + 1 mM iodoacetate and 10 mM leucine + 2 mM fluoroacetate do not produce a significant increase in cytosolic Ap_4A and Ap_3A levels. 5°) 20 mM arginine, 10 mM KCl and 100 µM Tolbutamide do not produce a significant increase in cytosolic Ap_4A and Ap_3A levels. Conclusions: In pancreatic islets, DPs synthetic pathway requires the metabolization of fuel secretagogues. These findings point out to DPs as new linking molecules between nutrient metabolism and insulin secretion.

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10-15

INFLUENCE OF THE COVALENT COUPLING METHODS ON THE REACTIVITY OF IMMUNOLATEX BEADS. I. Miraballes-Martínez[1], A. Martín-Rodríguez[2] and R. Hidalgo-Álvarez.[2]

Purpose: The antigen-antibody reaction can be detected in a macroscopic way by means of immunolatex beads. Latex beads act as support structures which can be used as sites for the physical or chemical attachment of biological macromolecules. In order to improve the immunoassay results, two different covalent coupling methods are investigated and the stability of the latex-IgG complexes is compared.

Methods: Sensitized latex particles were obtained by immobilization of IgG (anti HSA) by covalent binding to carboxylated and chloroactivated latexes. The stability of the IgG-latex complexes were evaluated by size determinations carried out by Photocorrelation Spectroscopy.

Results: The IgG-carboxylated latex complexes did not present the necessary colloidal stability to be useful as reagents in immunodiagnostic tests. The IgG chloro-activated latex complexes were stable under different conditions. High stability of this immunolatex, before the agglutination reaction, was obtained by working at low ionic strength during the sensitization process. Also was found high reactivity of these complexes under physiological conditions.

Conclusions: Immunolatex obtained by covalent coupling of IgG on chloroactivated latex presents the appropriate stability and reactivity to use as reagent in diagnosis tests.

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10-16

ISOLATING AND CHARACTERIZING DIFFERENT TYPES OF BACTERIA USING DIELECTROPHORESIS. M. Mohamed and A. Nashaat.

Dielectrophoresis is the movement of particles in nonuniform a.c. electric fields. Suspensions of bacteria were used as model systems to investigate the electrokinetic behavior of bioparticles subjected to travelling electric fields using microelectrodes. When nonuniform electric fields are created between microelectrodes, bacteria will redistribute themselves around the electrodes. The force holding the bacteria in place depending on the frequency of the applied nonuniform electric fields, the electrical properties of the bacteria and the conductivity of the suspending medium. Steric drag forces produced by a gentle fluid flow in the chamber can be used to separate different types of bacteria as well as to see if they were viable or not by selectively lifting bacteria from potential energy wells produced by the electric field. The effect of electromagnetic field on bacteria characteristic was studied using this new dielectrophoresis technique.

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10-17

13-C NMR SPECTROSCOPY OF ACETATE AND PYRUVATE METABOLISM IN SOLANACEOUS CELL SUSPENSIONS. RELATION WITH ALKALOID BIOSYNTHESIS. F. Mesnard, D. Marty, F. Gillet-Manceau, M.A. Fliniaux, J.P. Monti.

The levels of tropane and tobacco alkaloids are often very low and even sometimes go undetected in Solanaceous cell suspensions. One reason could be that the key enzymatic systems between primary and secondary metabolisms are lacking or inhibited in plant cell cultures. Coupled with the use of labeled compounds, such as [2-13C] acetate and [3-13C] pyruvate, carbon-13 nuclear magnetic resonance (NMR) spectroscopy can provide an extremely powerful technique for characterizing metabolic pathways. Cultures of *Nicotiana plumbaginifolia* cells that do not produce alkaloid were supplemented with the labeled compounds. It results in a considerably labeling of glutamine compared to glutamate. The data show a high activity of glutamine synthetase with a very low concentration or a lack of glutamate due to its storage in glutamine form. The results might be a first step towards explaining the non-biosynthesis of alkaloids because glutamate is a key amino-acid which yields to putrescine, precursor in alkaloid biosynthesis, via ornithine.

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10-18

SELECTIVE PERMEABILITY OF EXTRACELLULAR ENVELOPE OF THE MICROALGA SPONDYLOSIUM PANDURIFORME AS REVEALED BY EPR TECNIQUE. **C.S. Freire-Nordi.; A.A.H. Vieira and O.R. Nascimento**

This work was done to verify if the polysaccharide sheath of the microalga *Spondylosium panduriforme* has any role in the selective process of permeation and transportation of molecules into the interior of the cell. To obtain this kind of information we used the EPR technique and several spin labels of hydrophobic nature. To separate the effect of the polysaccharide sheath we removed it by ultrasonication treatment and the permeation measurements were done in these two algi conditions. The cultures of the alga were grown in WC medium under axenic conditions. We measured the time decay of the spin label until it enters inside the cell. The intensity decay was monitored as function of time and the time decay is around several minutes. The time decay of the cells with capsule were different and they could be arranged in order of increasing diffusion time as: Isothicyanate-Tempo, Phenyl-Imidazoline, Tempo, Tempol and Tempone. Tempamine and Maleimide did not permeate across the capsule. Diffusion times using cells without capsule were faster than those for cells with capsule. Tempamine migrated across the cell wall and membrane on removal of the capsule. Maleimide did not permeate across the cell wall and membrane too. We could conclude from these results that the capsule has a role of a selectivity medium probably due to an interaction of a polar nature involving hidrogen bonds.

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10-19

FUNCTIONAL AND ORIENTED IMMOBILIZATION OF NA^+,K^+-ATPASE ON PLANAR WAVEGUIDE SENSOR SURFACES.
M. Pawlak, E. Grell, E. Schick, D. Anselmetti and M. Ehrat

The controlled immobilization of membrane proteins/receptors on solid surfaces, under preservation of their functional activity, is a major challenge for systematic biophysical investigations as well as for biosensor applications, where the protein's sensitive and selective bioaffinity interactions with bulk ligands are exploited. In a first case, Na^+,K^+-ATPase, an ATP-driven ion pump, establishing the cell membrane potential, was used as a model system. Purified ATPase was prepared from dogfish rectal gland as a suspension of enzyme containing membrane discs (mean diameter ≅ 300 nm) with a concentrated protein:lipid ratio. A protocol was developed to immobilize the membrane discs on lipid-coated, hydrophobic metaloxide waveguide surfaces, yielding large and stable surface coverages of ≥50%. The different states of surface preparation and, in particular, the morphologies of self-assembled lipid films and associated membrane discs on the waveguide surfaces were characterized by tapping mode AFM in solution. The kinetics of membrane disc association during the surface preparation and following assays, probing the functional activity and orientation of the surface-immobilized enzyme, were performed by measuring specific changes of the surface-confined, intrinsic fluorescence of a FITC-labeled ATPase analogue, which was excited in the evanescent field of the planar waveguides. The specific K^+-binding of the enzyme, for instance, was measured with a binding characteristics comparable to bulk experiments, revealing a dissociation constant of K_D ≅ 200 μM. Only the comparison of results from different investigations, the characterization of bulk and surface-confined protein fluorescence, combined with AFM, allowed a clear and quantitative determination of enzyme function, orientation and location of affinity sites.

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10-20

MEASUREMENTS OF THE PRESENCE OF H_2 DISSOLVED IN URINE BY USING AN ELECTROCHEMICAL SENSOR
M.A. Esteso, R. Rodríguez-Raposo and J.J. Podestá

The intraintestinal production of hydrogen gas results from the fermentation of nonabsorbed sugars by anaerobic bacteria. The carbohydrate malabsorption in gastrointestinal diseases is accompanied by a higher development of such intestinal hydrogen. The lack of reproducibility for the useful quantitative tests limits the adequate measurement of this biological hydrogen which is eliminated through: 1) anal excretion, 2) absorption and transference to the portal circulation followed by lungs excretion and 3) the metabolism by other colonic bacteria.
In the present investigation we attempt to demonstrate that intestinal H_2 can be detected and measured dissolved in urine by using a novel electrochemical SPE sensor.
Our results on urine samples, obtained (with the application of an extremely simple protocol) for various healthy and intolerant patients are in accordance with those obtained by the classical clinic test of hydrogen excretion measurements in breath.

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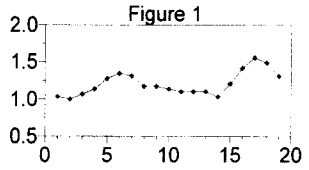
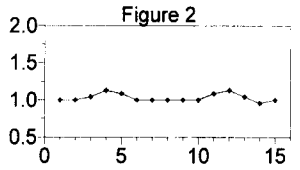
10-21

ETHANOL EFFECTS ON EPIDERMIS IN MULTIPLE ACUTE CONTACTS. STUDIES BY ^{31}P-NMR SPECTROSCOPY S. Sardón, J.L. López-Lacomba, J.A. Sánchez-Alcázar, and J. Ruíz-Cabello.

Topically applied compounds can contain potential skin irritants such as solubilizers, surfactants, penetration enhancers, preservatives and drugs. Ethanol is extensively used because of its properties as a liposoluble drug solvent and as a permeation enhancer. Nuclear Magnetic Resonance Spectroscopy is a non invasive technique which is helping us to develope metabolic studies on intact tissues. ^{31}P spectroscopy let us following the most representative energetic metabolites (NTP's and phosphocreatine) as well as phospholipids metabolites (phosphomonoesters and phosphodiesters). The current work has been developed using pig epidermis isolated and kept alive under perfusion with an isotonic, phosphate free and enriched media. The following of the metabolite levels along different experiments, have shown a reversible increase in PME, and a decrease in phosphocreatine levels during the acute ethanol perfusion at a final concentration of 1% (v/v). Multiple contacts experiments were developed and enhanced effects were recorded in those experiments in which the lag time betweeen perfusions with the agent was of eight hours (figure 1). However, experiments with a lag period of four hours did not show any statistically change (figure 2).





Relative intensity height of phosphomonoesters are plotted vs time in hours. Ethanol at 1% final concentration was used along shaded hours.**Figure 1.-** Lag period between shaded hours is eight hours.**Figure 2.-** Lag period between shaded hours is four hours.

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10-22

SPIN COUPLING IN COMPOUND I MODELS OF PEROXIDASES: EPR AND MÖSSBAUER SPECTROSCOPY ON (Fe(IV)=O)$^+$-PORPHOLACTONES. V. Schünemann[1], M. Müther[1], A.X. Trautwein[1], E. Bill[2], R.N. Austin[3], K. Jayaraj[3], A. Gold[3], J. Terner [4], D.Mandon[5] and R. Weiss[5].

H_2O_2 as a toxic side product in the respiratory chain is used up as an oxidans in peroxidase as well as in catalase reactions. In case of iron containing enzymes the active site is a heme-bound Fe center. The oxoferryl porphyrin π-cation radical (compound I) and the oxoferryl porphyrin (compound II) transients are key intermediates in the catalytic cycle of these enzymatic reactions.

EPR and Mössbauer spectroscopy are effective tools to study the spin coupling strength J of ferryl iron (S=1) and porphyrin radical spin (S'=1/2) in compound I intermediates. Parallel spin coupling has been found in catalases, weakly parallel coupling in horseradish peroxidase and antiparallel coupling in chloroperoxidase.

Considerably effort has been undertaken to model chemically compound I intermediates. However modelling of compound I analogues exhibiting antiparallel spin coupling has not been successfull up to this date. Nevertheless, we were able to prepare a weakly parallel coupled compound I analogue by oxidation of a ferric porpholacton with m-chloroperbenzoic acid (m-CPBA) at -80° C.

A spin-Hamiltonian analysis (including Heisenberg exchange J·S·S' and zero field splitting) of both the EPR and the Mössbauer data (the latter obtained at 4.2K and in fields from 0.02 up to 7T) leads to $J=11cm^{-1}$ which is among the lowest found so far.

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10-23

SYNTHESIS AND SPECTROSCOPIC STUDY ON POLY(DIORGANOAMINO)PHOSPHAZENES AS THE POLYMERIC MATRIX OF MEDICAL LIGANDS. A.Sułkowska and W.Sułkowski

Poly(dichloro)phosphazenes, as a starting material, was synthesized in bulk polymerization process at 523K according to our original methods. Poly(diorganoamino)phosphazenes were synthesized according to the optimized method described earlier. For optimization of reaction conditions and possibilities of full chlorine atoms substitution, the initial study was performed for a hexachlorocyclotriphosphazene. The full substitution of chlorine atoms is necessary due to the application of phosphazene derivatives as a medical agent carriers. The n-butylamine, piperidine and imidazole were used as organoamino substituents. The 1-ethanolo-2-methyl-5-nitroimidazole (metronidazole) as a medical carrier was introduced into the polymeric matrix. The structure of synthesized cyclo(diorganoamino)-, poly(diorganoamino)phosphazenes, and polymers of metronidazole were determined using the results of elemental analysis, (where zero content of chlorine shows on full substitution of chlorine atoms), the IR, ^{1}H NMR, and ^{31}P NMR spectra. Only one δ_P signal of 2.0, 3.9 and 5.8 ppm respectively for poly(di-*n*-butylamino)phosphazene, poly(dipiperidino)phosphazene and poly(diimidazolo)phospazene was observed. Spectra of poly(metronidazolo-n-butyloamino)phosphazene and poly(metronidazoloimidazolo)phosphazene show only two δ_P signals of 2.0, 14.0 and 5.9, 12.7 ppm respectively, which confirms the assumed structure of polymers. The T_g and T_m values equal to 313K and 410K for poly(di-n-butyloamino)phosphazene and respectively 308K and 433K for poly(dipiperidino)phosphazene were also determined. By synthesis of these polymers, the possibility of introduction of low molecular biology active agent into polyphosphazene chain was shown.

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10-24

COMBINATION OF PET MEASUREMENTS WITH IMAGE REGISTRATION AND KINETIC MODELLING FACILITATES OBSERVATION OF DRUG EFFECTS Trón, L., Balkay, L., Emri, M. and Márián, T.

Cavinton (vinpocetine: ethyl apovincaminate) is frequently used in the therapy of patients with cerebrovascular disorders. Among other effects vinpocetine has been shown to dilate cerebral blood vessels, and increase the cerebral glucose uptake, however these findings were not confirmed by modern experimental methods. A dynamic FDG-PET study was therefore carried out to detect any changes in the glucose metabolism of the brain of stroke patients following a single dose of 20 mg of Cavinton, administered IV. For these purposes difference images were constructed by subtraction of the appropriate static images measured before and after vinpocetine treatment. A characteristic pattern related to the geometry of the stroke area was found on these difference images but presubtraction registration of the appropriate images eliminated those pattern. Analyzing the dynamic data by the three compartment model of FDG accumulation revealed in Cavinton induced changes in the kinetic constants of this model. A significant increase in the value of the characteristic rate constant describing transport of FDG between the vascular and the intracellular space was detected within the regions not affected by stroke. In addition, a similar significant increase was observed in the value of rate constant determining transport from the intracellular to vascular space within the stroke affected area. Our data show that image registration and kinetic analysis of PET data can reveal otherwise unnoticed effects of drug treatments.

This study was supported by OTKA grants T 16149 and T 16150.

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10-25

EFFECT OF STORAGE ON ELECTRICAL IMPEDANCE OF APPLE TISSUES. E. Vozáry, P. László and P. Sass.

The aim of this work was to determine that change in the apple tissue during storage of 7 months, which can be detected by electrical impedance measurements.

The magnitude and the phase angle of complex impedance in the frequency range of 30Hz to 1MHz were measured with a HP 4284A precision RLC meter. The voltage on the samples was 1,0V. The two electrodes were punctured across the equator into the whole apple with skin and into the fresh apple flesh produced by cutting a spherical calotte of 1cm thickness from the whole apple.The distances between the electrodes were 2, 5, 10 and 20mm. The impedance spectra of thin apple slices of 3.5mm x 3.5mm x30mm were also measured .

A simple method was elaborated to calculate the impedance of the electrodes, the apple skin and the apple tissue. The impedance of apple tissues were approached by impedance value of equivalent electric circuit, the elements of which can represent the resistance and the capacity of the cell wall, the cell membrane and the citoplasm.

Changesin the resistance of the citoplasm can not be observed during the storage , but a significant decrease can be seen in the resistance of the cell wall. This change can be explained by the increasing number of ions in the apoplasm during the ripening in the storage period. This fact is supported by the result of other physiological experiments. The decrease in the resistance of low frequency can be realized by the changes in the resistance of cell walls too.

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10-26

COMPARATIVE STUDY OF TOPOGRAPHICAL ANALYSIS METHODS. F. Wallet, C. Dussert.

Biological entities at various scales (macromolecules, subcellular structures, cells, tissues) are often related each to the others through complex interactions. In order to study cooperative behaviour of these entities a way of increasing use is topographical analysis : the quantification of the spatial patterns formed by the entities considered as points.

We compared five methods of topographical analysis in term of discriminant power, stability of parameters, methodological bias and algorithmic. We tested five methods (nearest neighbour distribution, radial distribution, Voronoï paving, quadrat count, minimal spanning tree graph) which generated nine parameters on four simulated models (random point process, hardcore model and two cluster models: big and small clusters) and on experimental cellular models. Multi-parameters methods are really more discriminant than mono-parameter ones. Methodological bias (features computations, parameters dimensionality and normalization, sampling windows and border effects) are taken into account. We classified the five methods in term of algorithmic complexity: radial distribution is the slowest method. The method which offers the best discrimination power and stability seems to be the minimal spanning tree graph edge length distribution.

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10-27

HIGHFIELD EPR OF FREE RADICALS FROM DNA AFTER IRRADIATION WITH X-RAYS OR HEAVY IONS. B. Weiland, J. Hüttermann and J. van Tol.

A large number of radicals from irradiated DNA model substances have been detected by X-band (9.5 GHz) and Q-band (34 GHz) EPR spectroscopy. All are characterized by small g-anisotropy and often have similar spectral shapes at these frequencies. In order to enhance the identification of the strongly overlapping component spectra in DNA by g-factor separation, we applied EPR at 245 GHz (8.7 T). Spectra were taken from nucleotides and DNA in different environments. We also searched for the influence of intercalating (proflavine) or non-intercalating agents (riboflavine, azomycine, potassium hexacyanoferrate, cysteine) in low concentrations on the radical formation in DNA after irradiation with X-rays or heavy ions at low temperature.

Ten radical components from nucleotides were isolated and simulated by comparison with X- and Q-band single crystal data from the literature. For DNA the highfield spectra confirmed the initial presence of at least three primary radicals, the oxidized guanine as well as the reduced cytosine and thymine species. At higher humidity the reduction of the cytosine seemed to be favoured above thymine according to its higher electron affinity. The yield of the oxidized guanine base was found to be reduced in frozen aqueous solutions, indicating different mechanisms of radical formation or stabilisation. Intercalating and non-intercalating agents which act as electron scavengers were effective in preventing the formation of the reduced DNA radicals after X-irradiation as well as after heavy ion bombardment. The thiol cysteine, however, did not show much influence.

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10-28

PARA- AND SUPERPARAMAGNETIC RELAXATIONS DETECTED BY NUCLEAR RESONANT FORWARD SCATTERING. H. Winkler[1], O. Leupold[2], S. Schwendy[1], W. Meyer-Klaucke[1], H.D. Rüter[2], D. Mandon[3], A.X. Trautwein[1] and R. Weiss[3].

Measurements of the time dependence of nuclear resonant forward scattering (NFS) of synchrotron radiation can be regarded as Mössbauer spectroscopy in the time domain. Therefore, one expects that it presents a new method to study dynamic processes like paramagnetic and superparamagnetic relaxations in condensed matter.

A metal ion complex with unpaired spins which couple to a total spin S constitutes a paramagnetic system. Under the influence of ligand fields (zero-field interaction) and applied magnetic fields (Zeeman interaction) the (2S+1)-fold degeneracy of the paramagnetic state is lifted. Paramagnetic relaxations take place in the form of random transitions in time between these spin (sub-)states.The mechanism of spin-lattice relaxation can be observed in every detail as a function of temperature in the NFS spectra, e. g., of the 'picket-fence' porphyrin $[Fe^{II}(CH_3COO)(TP_{piv}P)]^-$, a high-spin iron(II) complex.

Superparamagnetism is a well-known phenomenon present in magnetic single-domain particles. It is in practice often used to estimate the size of such particles. Bacterioferritins (BF) contain mineral cores, which in physical terms are magnetically ordered particles exhibiting superparamagnetism. This means that below the so-called blocking temperature the direction of magnetization stays fixed for time intervals longer than the Larmor precession period, while above it flips at a rate so fast that the magnetization appears to be averaged to zero. The spectra of BF from *S. olivaceus* with a blocking temperature of about 8 K show this property by the transition from fast to slow beating patterns. -- Financial support of this work by the BMFT (grant 05 643FLA) and the Alexander von Humboldt foundation is gratefully acknowledged.

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