## *Posters*

*- Lipid-protein interactions -*

## 1-1

### Construction of polymer-tethered phospholipid bilayers for protein-membrane interaction studies.

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A general method in which tethered membranes are anchored to the solid support using bi-functional lipid-polyethylene-glycol molecules was developed. The hydrophobic tail of the lipid-PEG molecules is mixed within the lipid membrane inner layer while their hydrophylic counterpart is covalently linked to the support. Proteins (such as myristoylated proteins, porin or CyaA) biological functions are studied using these biomimetic membranes. Molecular construction of the membrane models are monitored using surface plasmon resonance apparatus while mere binding kinetics are determined using the fluidic potentialities of the Biacore instrument. Characterisation of the tethered bilayer architecture was achieved by FRAP and AFM measurements. The combination of optical characterisation of the tethered bilayer architecture, their functional assessment (binding and/or channeling properties) by the simultaneously applied optical and electrochemical techniques and the correlation of structural/functional parameters will lead to an unprecedented tool in model membrane research.

## 1-2

### Polymer-liposome association induced by electrostatic interactions

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The association of water-soluble vinyl polymers to dimyristoylphosphatidic acid unilamellar vesicles is investigated as a function of pH, ionic strength and temperature using steady-state fluorescence spectroscopy and viscometry. Poly(2-vinylpyridine) and poly(4-vinylpyridine) fluorescence data are converted to association isotherms and discussed in terms of both binding and partition models. The results of this work support previous suggestions: (1) in the case of polyions the inclusion of the activity coefficient in both models is essential; (2) the parameters calculated using both different theoretical approaches are directly compared by the proposed relating equation. The excellent agreement of steady-state fluorescence and viscometry results allows to assume a model for the polymers approaching and for their adsorption on the surface, where the length of hydrophobic chain as well as the position of the N atom in the pyridinium ring play an important role. The transition temperatures for the interaction of both polyvinylpyridines with dimyristoylphosphatidic acid unilamellar vesicles at pH 3.5 and ionic strength 0.026M are determined from experimental and theoretical data. Aqueous size-exclussion chromatography is proposed as a major technique for monitoring the interactions between liposomes and polyvinylpyridines. The theoretical treatment is an elegant method because both Gouy-Chapman and Debye-Huckel formalisms apply the Poisson-Boltzmann equation to calculate the influence either of a plane charged surface or between two charged spheres over the structure of the adjacent ionized liquid. In both cases, the magnitude of the medium that has physical meaning is the Debye length.

## 1-3

### Study of membrane destabilisation by penetratin peptides

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Penetratin is a 16-residue peptide (43-RQIKIWFQNRRMKWKK-58) derived from the third helix of the Antennapedia homeodomain, a Drosophila transcription factor. This peptide is suitable as a vector for cellular internalisation of hydrophilic molecules, through a translocation mechanism that is yet not fully understood. We previously showed that Penetratins strongly associate with negatively charged liposomes [Christiaens et al., 2002, Eur. J. Bioch. 269: 2918-2926]. In this work, the contribution of specific positively charged residues to membrane destabilisation and cellular uptake was investigated by comparing the properties of R52A/K55A, K46A/R53A, K46A/K57A and R53A/K57A variants to the WT Penetratin peptide. Trp fluorescence titrations pointed out that these new variants have lower affinity for negatively charged vesicles. In spite of this lower affinity, the R52A/K55A and R53A/K57A variants induce more calcein leakage than WT Penetratin. WT Penetratin induces vesicle aggregation, while the double Ala variants do not. Aqueous and lipid phase Trp fluorescence quenching experiments showed that the R52A/K55A and R53A/K57A variants insert more deeply into the lipid bilayer than the WT peptide. These results suggest that the extent of calcein leakage by the Penetratin peptides is related to the depth of insertion in the lipid bilayer, while the mean hydrophobicity and the amphipathic helical moment are key factors for membrane destabilisation. The influence of the above mutations on the internalisation efficiency of NBD-labeled peptides in MDCK cells was studied by flow cytometry. The extent of internalisation of the K46A/R53A variant is decreased to about 15% of WT, whereas the R52A/K55A, K46A/K57A and R53A/K57A variants have an internalisation efficiency of 50% of WT Penetratin. These results thus suggest that the mean hydrophobicity and amphipathic helical moment are critical for model membrane destabilisation, while they are not the only factors modulating the cellular uptake efficiency.

## 1-4

### Membrane restructuring by bordetella pertussis adenylate cyclase-toxin, a member of the RTX toxin family

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Adenylate cyclase toxin (ACT) is secreted by Bordetella pertussis, the bacterium causing whooping cough, ACT is a member of the RTX (repeats in toxin)family of toxins and, like other members in the family, it may bind cell membranes and cause disruption of the permeability barrier, leading to efflux of cell contents. The present work summarizes studies performed on cell and model membranes, with the aim of understanding the mechanism of toxin insertion and membrane restructuring leading to release of contents. ACT does not necessarily require a protein receptor to bind the membrane bilayer and this may explain its broad range of host cell types. Toxin effects are however supressed by poly(ethylene glycol) grafted onto the membrane surface. Ca2+ is not required for ACT insertion into a lipid monolayer at the air water interface, but efflux occurs only in the presence of mM Ca2+. Release of aqueous vesicular contents is enhanced by the presence of negative curvature lipids, and inhibited by lipids favouring positive curvature. These results strongly suggest that ACT-induced efflux is mediated by transient non-lamellar lipid structures. This is confirmed by the experimental observation that efflux is accompanied by transbilayer ("flip-flop") lipid motion in the same time scale.

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## 1-5

### Site-directed mutants at HIS859 reveal the importance of this residue in the activity and calcium binding properties of escherichia coli a-haemolysin

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E. coli alpha-haemolysin is a cytolytic exotoxin secreted by pathogenic strains. This toxin is the best characterized member of a family of widely disseminated toxins known as RTX toxins for a series of Gly-Asp rich repeats in the carboxy-terminus of their structural proteins. They also share other features, an N-terminal hydrophobic domain organized as amphipathic helices, a fatty acylation domain where the essential posttranslational activation by amide linked palmitoylation takes place and a C-terminal export sequence. Though necessary, the haemolysin activation through the acylation of its Lys 564 and 690, is not enough for the toxin to be fully active, as in the absence of calcium the acylated toxin in inactive. An additional step is indispensable for the hemolysin to be lytic. This second activation occurs when the toxins binds calcium, which induces in the protein a conformational change that increases the surface hydrophobicity of the protein, thus facilitating its irreversible bilayer insertion as intrinsic membrane protein. In a previous work we had studied the binding characteristics of this cation and proposed that the repeat of sequence SGYGHHIID extending from residues 855-863 could be a good candidate for exploring its possible implication in calcium binding, due to the presence of an His in the natural position of an Asp in the rest of the repeats. Thus, in the present work we have constructed site directed mutants at His 859 to analyze the implication of His residues in cation binding and thus in the hemolytic activity of the toxin.

## 1-6

### Cytochrome c in lipidic environment: structural modifications and self-exchange electron transfer

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Cytochrome c is a hemeprotein with strong interactions with the internal mitochondrial membrane. These protein-lipid interactions are believed to be involved in the regulation of the electron transfer rate and the activation of the caspases at the early stage of the apoptosis. In the present report, we describe the results of the proton NMR of the cytochrome c in micellar and liposome environment. Profound structural modifications are observed with rupture of the iron-methionine bound. With a low micelle-protein ratio, a high rate of ligand exchange at the sixth binding site of the iron is consistent with the absence of any resolved NMR signals. With a high micelle-protein ratio, a low spin to high spin transition of the iron is observed. According to previous works with different spectroscopies the protein is not fully unfolded. Modified cytochrome c can be further analysed using the free binding site available in both oxidized and reduced states. Binding of exogenous ligands on this myoglobin-like structure can be used to stabilized the active site. Carbon monoxide gives stable complex on the reduced form, whereas cyanide is able to complex both the ferric and the ferrous forms with high affinities. Using cyanide, the self-exchange electron transfer rate of micellar cytochrome c can be determined by NMR experiments.

## 1-7

### Fluorescence study of hemoglobin interaction with phospholipid vesicles

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Lipid-associating properties of hemoglobin (Hb) have been widely studied in several main aspects concerning, particularly, physiological role of Hb binding to erythrocyte membrane, Hb-induced oxidative membrane damage, hemosome stability, and elucidating general features of protein-lipid interactions. The present study was undertaken to gain further insight into the mechanisms of Hb-lipid binding process. Special attention was given to evaluating contribution of electrostatic phenomena to association and dissociation kinetics of Hb-lipid complexes under conditions of varying pH, ionic strength, and lipid bilayer surface charge density. The interaction of Hb with lipid vesicles composed of phosphatidylcholine (PC) and cardiolipin (CL) was examined by measuring the efficiency of resonance energy transfer from a membrane-incorporated anthrylvinyl phospholipid derivative (AV-PC) to the heme groups of Hb. Hb-induced lipid peroxidation was suppressed by addition of free radical scavenger (thiourea). It was found that i) the process of Hb interaction with lipids includes at least three kinetic stages whose rates tend to increase with increasing CL content from 10 to 40 mol%; ii) the fastest stage (< 30 sec) corresponds to formation of protein-lipid electrostatic contacts, while the slower ones (characteristic times 0.4-0.8 and 6-10 min) are assumed to reflect the protein penetration into the bilayer interior; iii) elevating ionic strength after 30 min sample incubation resulted in partial dissociation of Hb from lipid vesicles exhibiting two main kinetic phases (characteristic times 0.4-0.5 and 4-16 min) that can be attributed to the protein movement across the bilayer and detachment from the membrane surface.

## 1-8

### Pore-forming activity of antimicrobic lipodepsipeptides from pseudomonas SPP on model membranes

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Lipodepsipeptides produced by many Pseudomonas species are divided into 2 groups called mycins and peptins. Syringomycin, syringotoxin, pseudomycin and cormycin belong to the first group: they are composed of 9 residues, cyclized in a ring. Bigger syringopeptins (22 and 25), and corpeptin belong to the second group: they are composed of either 22 or 25 amino acids, cyclized in a 7 residues ring. The recently purified cormycin and corpeptin from P. corrugata share some structural features and biological properties with the better characterized LDP from P. syringae. Mycins and peptins significantly contribute to bacterial pathogenesis, and are also active on the plasma membrane, but may have different target organisms. We compared the activity of all LDPs on red blood cells from different origin (human, rabbit and sheep RBC) and on liposomes of different lipid composition. All peptides induced RBC hemolysis, sheep erythrocytes showing higher stability, and leakage of calcein from liposomes. Electrophysiological experiments on planar lipid bilayers demonstrated that the process of membrane destabilization is related to the formation of ionic pores. Mycins are more hemolytic than peptins, the extent of permeabilization was dependent on the liposome composition. In particular, mycins show a preference for sterol containing liposomes, whereas peptins are more active if sphingomyelin is present. (Sponsored by PAT Fondo Progetti-Project Agribio and MIUR)

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### Drug influence on the thermotropic phase transition of phospholipid/drug model-membranes.

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The temperature-induced fluidity of the lipid constituents is one of the most important factors associated with drug action. The changes in the main phase transition temperature of model membrane induced by some drugs such as gramicidin, penicillin, nifedipine were estimated by fitting the wavenumbers of CH2 symmetric and asymmetric stretching modes from FTIR spectra of DPPC/drug and DPPG/drug vesicles. Experimental data were obtained in a large interval of temperatures [25-70] Celsius degrees. The main phase transition temperature has been estimated by fitting experimental data with exponential functions. The main phase transition temperature decreases with about 1-3 Celsius degrees in the phospholipid vesicles containing gramicidin and penicillin and increases with about 2 Celsius degrees in the case of nifedipine-containing model membranes. By decreasing the main phase transition temperature of the phospholipids, the drugs can penetrate through biological membranes in the inner compartment of the cells. Nifedipine induces a sort of rigidity in the vicinity of the end of the acyl chains, increasing temperature of the main phase transition with about 2 Celsius degrees, while gramicidin and penicillin produce a destabilization of the acyl chains and decreases the main phase transition. The mathematical model permits to establish the coordinates of the main phase transition as function of the drug concentration and to understand the specific way of the drug penetration in the model-membrane. The proposed mathematical model permits quantitatively express the drug action on the cell membrane, giving the limits of temperature and drug concentration in which the membranes are stable systems.

## 1-10

### Prodan fluorescence influenced by high density lipoproteins and alcohol

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The moderate alcohol consumption is proven to be associated with a reduced risk of coronary heart diseases (CHD). It is accepted that plasma lipoproteins are strongly involved in pathogenesis of atherosclerosis and therefore it would be of interest to look at their interactions with alcohol. Determined increase in the level of high-density lipoprotein(HDL) correlated with alcohol consumption might contribute to this reduction. However, elevated HDL levels are measured in heavy drinkers, but the antiatherosclerotic behavior of HDL was not effective. Therefore, it would be of interest to study the impact of alcohol concentrations on HDL behavior. We have studied the impact of ethanol concentrations on the structure of two human HDL subfractions, HDL2 and HDL3, with particular interest on lipid-protein interactions. The alcohol concentrations were chosen to mimic the concentrations in the blood stream. To follow the modulations in the lipid-protein interface induced by ethanol the fluorescence spectroscopy was applied on unlabeled, natural, and on HDLs labeled with membrane probe 6-propionil-2-dimethilaminonaftalen (Prodan) which locates in the head group region of phospholipid near the lipid-water interface. Spectra of Prodan are highly sensitive to the polarity and the mobility properties of the environment. The comparison of optical behavior; intensity changes, shift in wave lengths, steady state anisotropy, quenching rates; of intrinsic protein probe, tryptophan,and lipid probe Prodan upon alcohol addition indicated the opening of the protein structure and decrease of the ordening in the lipid moiety of HDL particles. The conformational changes have been confirmed by fluorescence resonance energy transfer measurements (FRET).

## 1-11

### Shape changes of giant unilamellar vesicles of phosphatidylcholine induced by a de novo designed peptide interacting with its membrane interface

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Reversible binding of proteins and peptides on the surface of lipid membrane regions in biomembranes plays important roles in cells. In this study, we investigated binding of peptides on electrically neutral lipid membranes such as phosphatidylcholine (PC). At first, we designed and synthesized a peptide, WLFLLKKK (peptide-1), which has positive charges and a segment partitioned into the electrically neutral lipid membrane interface, and investigated its effects on the stability of PC membranes. First, we investigated its effect on intermembrane interaction of multilamellar vesicle (MLV) of palmitoyloleoyl-PC (POPC). Its spacing increased greatly as peptide-1 concentration increased, but high concentration of NaCl reduced its spacing, indicating that the increase in its intermembrane distance is due to electrostatic repulsion. Secondly, effects of peptide-1 on shapes of giant unilamellar vesicle (GUV) of dioleoyl-PC (DOPC) were investigated using phase-contrast microscopy, because observation of shape change of GUV has been recently considered as a high-sensitive method detecting interactions between substances and lipid membranes (Biochim. Biophys. Acta, 1564, 173-182, 2002; ibid, 1561, 129-134, 2002). The addition of 5 micro-M peptide-1 through a micropipette near a DOPC-GUV induced several kinds of shape changes; e.g., a discocyte was changed to two-spheres connected by a neck, and small vesicles were budded into the outside of the spherical GUV. These results indicate that peptide-1 can be partitioned into the PC membrane interface and affect greatly its structure and property. Effects of other peptides on PC membranes are also reported in the Conference.

## 1-12

### Influence of the glycostructure of lps on pore formation by phosphoporin phoe of escherichia coli

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The lipid matrix of the outer membrane (OM) of Gram-negative bacteria is an asymmetric lipid bilayer composed of a phospholipid inner and a lipopolysaccharide (LPS) outer leaflet. Incorporated into this lipid matrix are the porins, which have a sieve-like function for transport or exclusion of hydrophilic substances. Performing electrical measurements on asymmetric planar bilayers composed of LPS on the one and a phospholipid mixture (PL) on the other side, we have studied the role of LPS, in particular that of the saccharide chain, in pore formation by phosphoporin PhoE of E. coli. The pore formation rate of PhoE added to the PL side of LPS/PL membranes depended on the length of the saccharide chain of LPS from various strains of Salmonella minnesota, whereas only slight differences for LPS from various deep-rough mutants were found. The efficiency of pore formation increased with increasing length of the saccharide moiety. Furthermore, voltage gating of PhoE was strongly dependent on the composition of the lipid matrix. For LPS from various rough mutants, the voltage necessary to induce pore closure was higher than the transmembrane voltage across the OM. Therefore, voltage gating plays only a minor role in the regulation of the transmembrane electrochemical potential across the OM. For PL/PL membranes, the voltage necessary to induce pore closure was less than the transmembrane voltage across the inner membrane. Thus, voltage gating may serve as protection mechanism preventing the inner membrane from a short circuit by accidentally incorporated porins. (S. Hagge et al., JBC, 2002)

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### The interaction of human plasma low density lipoproteins with high molecular weight heparin

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Low density lipoproteins (LDLs) are the main cholesterol carriers in human plasma directly involved in the development of atherosclerosis. They exhibit interesting structural complexity with the surface monolayer organization of mainly phospholipid molecules in which the amphipatic apolipoprotein B100 (apoB) is embedded surrounding the hydrophobic core of apolar lipids. Heparins are sulphated glycosaminoglycans of widespread clinical usage for its anticoagulant and antithrombotic activity. Their characteristic is heterogeneity in chain lengths and the sulphation degree. The aim of this study is to investigate the interaction of LDL and the high molecular weight heparin at the level of LDL tertiary structure since previous research has established that peptides of apoB obtained by tryptic and peptic digestion (rich in arginines and lysines) bind high molecular weight heparin. Intrinsic fluorescence spectroscopy is employed to obtain information on apoB structural rearrangements induced by heparin binding. In order to describe the interaction, the stability constants and the number of binding sites are calculated for 298 K and 310 K and ionic strengths of 0.01 and 0.2. The results are discussed in the framework of the previously obtained data on apoB secondary structure content at the same experimental conditions. The changes in protein and ligand dynamics have been followed by fluorescence anisotropy measurements. Time resolved fluorescence spectroscopy has been used to determine the effect of heparin on the intrinsic fluorescence lifetimes. By computational analysis of the apoB primary structure, the binding sites for heparin on the apoB are proposed.

## 1-14

### A novel spectrophotometric approach for monitoring structural rearrangements of human plasma low density and high density lipoproteins

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A novel spectrophotometric assay for monitoring structural rearrangements of surface and core of native low density lipoproteins (LDL) and high density lipoproteins (HDL3) has been developed. The approach is based on the analysis of the visible light absorbance maxima of lipoproteins assigned to beta-carotene situated in the lipoprotein hydrophobic core. The signal sensitivity has been tested through conformational changes of lipoproteins induced by ionic strength and temperature as well as caffeine binding to LDL and HDL3. The beta-carotene signal of the LDL particle appeared to be sensitive to ionic strength increase, caffeine binding and temperature induced phase transition of the LDL core. Stability constants for caffeine binding to LDL under different ionic strength conditions estimated by SPECFIT global analysis are of the same order of magnitude. HDL3 beta-carotene signal displayed lower sensitivity to ionic strength and caffeine concentration increase but can without a doubt be applied for ligand binding studies. The beta-carotene absorbance has been chosen due to its numerous advantages. It is intrinsic to the lipoprotein species and sensitive to structural rearrangements induced. No danger of spectral overlapping and artefacts is present since it is situated in the visible spectral region where most of other species present in human plasma do not absorb light. It represents a potential for development of a method for monitoring LDL changes directly from plasma.

## 1-15

### Characterisation of the interactions of biological peptides with artificial membrane-microdomains and cellular membranes; kinetics and imaging

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The interactions of the signal or leader peptide of cytochrome c oxidase (p25), and the fusion peptide domain of human immunodeficiency virus glycoprotein 41, gp41FD, with model and cellular membranes are outlined. A series of studies with both artificial membrane systems as well as with cellular membranes and living cells were performed including fluorescence imaging and kinetic determinations to determine the nature of peptide-membrane interaction. Some of these studies involved use of fluorescent probes such as the membrane electrostatic surface potential sensor, fluorosceinphosphatidylethanolamine (FPE). This was utilised, in conjunction with a stopped flow rapid mixing techniques as well as a fluorescence imaging system to study respectively, the very early peptide-membrane interactions and the spatial localisation of such interactions. Ordered lipid microdomains were introduced into model membranes with varying degrees of cholesterol and labelled with FPE. It was observed that increasing cholesterol content of the membranes led to reduced rates of binding and insertion of p25 and a reduction of the affinity as compared to those found with mitochondria. Quantification of fluorescence images of cholesterol containing PC bilayers indicated that membrane insertion of gp41FD was less favourable within the cholesterol-ordered lipid microdomains than in more fluid PC bilayers. It was found however, that the former structures promoted conformational cooperative behaviour of gp41FD that was identical to that found ex vivo (ie. within T cells). These observations will be discussed in context of the biological roles of these and related peptides.

## 1-16

### Interaction of alzheimer amyloid-beta peptide with membranes monitored by DSC, CD and biological mas NMR

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Amyloid-beta peptide (Ab) is a key substance in Alzheimers disease (AD), and characterized by its abnormal folding into neurotoxic aggregates. Since Ab comprises an extracellular and transmembrane domain, it can interact with neuronal membranes and exert cytotoxic effects. Here, we use biological solid state NMR in combination with differential scanning calorimetry and circular dichroism spectroscopy to study two types of interactions, protein insertion and surface association, between Ab1-40 protein and neuronal model membranes. Electrostatic association of Ab1-40 peptide via its charged amino acid residues to membranes with varying electrostatic surface potentials was monitored by 14N and 31P MAS NMR. Binding of Ab to membranes composed of DMPC and negatively charged DMPG lipids is mainly driven electrostatically, reflected in characteristic changes of the isotropic 31P chemical shift values for both lipids. In addition, the average orientation of the choline headgroup of DMPC, with its electric PN dipole, changes directly in response to the reduced negative membrane surface potential. The deviation in tilt angle of the PN vector relative to the membrane surface is manifested in the observed 14-N NMR quadrupole splittings and can be analysed semiquantitatively in a kind of "molecular voltmeter" approach. For 13-C labelled membrane-inserted Ab(1-40), high resolution solid state NMR provided structural details not only the secondary structure of its transmembrane part of the peptide but also for a predicted beta-hairpin structure in the hydrophilic part of the peptide. In addition, changes in isotropic 31-P chemical shift values for both lipids are consistent with a dual action of hydrophobic and electrostatic interactions upon insertion.

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## 1-17

**Mathematical modeling of prodan participation in solutions of serum HDL and ethanol**

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Membrane probe 6-propionil-2-dimethylaminonaphtalene (Prodan) was used to follow the changes introduced in the structure of lipid moiety of human serum high-density lipoprotein (HDL) upon addition of different amounts of ethanol. Prodan partitioning in solvent and phospholipids monolayer of HDL and its fluorescence behavior is highly sensitive on the microenvironmental polarity and fluidity. It preferably enters more fluent loosely ordered phospholipids phase. We followed the changes in fluorescence resonance energy transfer (FRET) from intrinsic protein probe tryptophan (apoA I) to Prodan (in lipid layer) and changes of Prodan steady-state anisotropy in solutions of different ethanol concentrations. Those results have been compared to the fluorescence behavior of Prodan in pure ethanol-water solutions of the adequate ethanol concentrations. The achieved data allow developing a model of Prodan behavior in HDL solutions, which would enable to explore the ethanol impact on the HDL structure.

## 1-18

**Activities of antimicrobial peptides studied by a lipid/polydiacetylene colorimetric assay.**

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Interactions between peptides and lipid membrane play major roles in determination of the action of antimicrobial peptides. We demonstrate applications of the newly developed lipid/polydiacetylene colorimetric assay for studying membrane interactions of polymyxin and polymyxin derivatives. The colorimetric platform facilitates evaluation of the degree of disruption of the lipid bilayer, the relative depth of penetration by the different antimicrobial peptides into the lipid assembly, relationships between peptide activity and membrane composition. We show that the information obtained by the colorimetric assay could shed light upon differences in the lytic activities of the peptides.

## 1-19

**Surfactant protein a decreases the binding of rough lipopolysaccharide (Re-LPS) to CD14 in the absence and the presence of LPS-binding protein (LBP)**

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LPS-binding protein (LBP) enables LPS's inflammatory activity by transferring LPS monomers from LPS aggregates to the pattern-recognition receptor CD14, and increasing the binding affinity of CD14 to LPS. In the alveolar fluid, surfactant protein A, a lipid binding protein involved in innate lung immunity, interacts with re-LPS and modulates re-LPS induced activation in vitro. However, the mechanism of SP-A/re-LPS interaction is not well understood. The aim of this study was 1) to characterize the interaction of SP-A with re-LPS by sedimentation, light scattering, and fluorescence analysis; and 2) to investigate whether SP-A inhibits the binding of LPS to either LBP, CD14, or LBP/CD14 using a photoactivable, radioiodinated LPS probe ([125I]-ADS-re-LPS). SP-A bound to FITC-re-LPS in a Ca2+ independent manner and, unlike LBP, could not disperse 3H-R-LPS aggregates. In the presence of calcium, SP-A induced LPS aggregation, measured by light scattering, and enhanced the sedimentation velocity of 3H-R-LPS aggregates. This process was likely mediated by specific Ca2+-dependent self-association of SP-A. Fluorescence analyses indicate that SP-A did not compete with LBP for the binding to FITC-re-LPS. Moreover, the presence of SP-A did not decrease the cross-linking of 125I-ASD-ReLPS with LBP. However, SP-A decreased the cross-linking of 125I-ASD-ReLPS with CD14, and inhibited the catalyzing activity of LBP to bind LPS to CD14. These studies support the idea that SP-A could be an important modulator in regulating LPS responses, likely altering the competence of the LBP/CD14 receptor complex.

## 1-20

**Conversion of endotoxically inactive pentaacyl endotoxins by cross-linked hemoglobin into a biological active conformation**

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Bacterial endotoxins (lipopolysaccharide, LPS) belong to the most efficient activators of the immune system which, however, may lead to pathophysiological effects such as septic shock. Here, the interaction of purified alpha, alpha - cross-linked hemoglobin (alpha, alpha-Hb) with an endotoxically inactive pentaacylated mutant lipopolysaccharide (pLPS) and the corresponding lipid A (pLA) was studied biophysically and the effects correlated with data from biological assays. Fourier-transform infrared spectroscopic and Zeta sizer experiments indicated an electrostatic as well as a non-electrostatic binding of alpha, alpha-Hb to the endotoxins with an increase of the inclination angle of the pLA backbone, with respect to the membrane surface, from 25 degree to more than 50 degree [1]. X-ray diffraction measurements indicated lipid A structure changes due to alpha, alpha-Hb interaction. Thus, in the absence of alpha, alpha-Hb the molecular shape of the pentaacyl samples was cylindrical, whereas in the presence of the protein the shape was conical [2]. Cytokine-inducing capability in human mononuclear cells was negligible for the pure pentaacylated compounds, whereas increased markedly in the presence of alpha, alpha-Hb. In the Limulus assay the pentaacylated samples were active a priori, and their activity was enhanced following binding to alpha, alpha-Hb, at least at the highest protein concentrations. The biological data can be understood by the reaggregation of the endotoxins due to alpha, alpha-Hb binding in a way that the endotoxin backbones are readily accessible for serum and membrane proteins. By using fluorescence resonance energy

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## 1-21

### A comparison of C2 domains from classical PKC as seen through DSC.

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The C2 domain is a membrane-targeting module found in a large number of signalling proteins. In classical PKC, these modules form independently folding domains that bind phospholipids in a Ca2+-dependent manner. The unfolding properties of classical PKC C2 domains were studied in the presence of different concentrations of Ca2+ and phospholipids using DSC. Tc of C2 domains were shifted to higher temperatures with increasing Ca2+ concentrations. The protection effect of Ca+2 allowed the observation of two different Ca2+-binding processes, which occurred at different Ca2+ concentrations, and PKC alpha and PKC gamma C2 domains showed a higher affinity than PKC betaII C2 domain. The presence of phospholipids enhanced this protection effect. In this case, the PKC gamma and PKC betaII C2 domains were more efficiently protected than the PKC alpha C2 domain. A very important effect occasioned by phospholipids, in the absence of Ca2+ was the widening of the transition leading to protein unfolding, but this effect was progressively eliminated by increasing concentrations of Ca2+. It was observed that DH increased with increasing Ca2+ concentrations and from the plotting of DH versus log [Ca2+] it was possible to detect as well the binding of Ca2+ with high affinity. Although the sequence of C2 domains from classical PKCs is highly conserved and the 3D structures are quite similar, the unfolding study presented here detect some differences about the Ca2+ affinity and structure and these differences might be related to the specific cellular localization and activation of each C2 domain.

## 1-22

### M13 coat protein selectivity towards phospholipids - a fret study

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Fluorescence resonance energy transfer (FRET) studies were performed on vesicles of various phospholipids with the major coat protein from bacteriophage M13 incorporated. The mutant T36C of this protein labelled with coumarin was used as a donor and a 7-nitrobenz-2-oxa-1,3-diazo-4-yl (NBD) fluorophore attached to the headgroup of DOPE was used as an acceptor. From previous work [1], it is found that DOPC bilayers offer perfect matching conditions for the membrane-bound form of the M13 coat protein. By using bilayers of different compositions it is possible to verify changes on the interaction between the NBD-DOPE probe and the labelled protein. Theoretical simulations developed with different models for phospholipid selectivity by the protein are compared with the experimental results. The FRET efficiency data were identical for M13 major coat protein incorporated in pure vesicles of both matching (di-18:1-PC) and not matching (di-14:1-PC, di-22:1-PC) phospholipids, suggesting a random NBD-DOPE probe distribution in all cases. However, an enhanced FRET efficiency was observed for di-18:1-PC/ di-22:1-PC mixtures, possibly due to a protein selectivity for the matching phospholipid. [1] F. Fernandes, L.M.S. Loura, M. Prieto, R. Koehorst, R.B. Spruijt, M.A. Hemminga, Dependence of M13 major coat protein oligomerization and lateral segregation on bilayer composition, Biophys. J., submitted.

## 1-23

### Optical manipulation of liosomes as microreactors

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In recent years liposomes have received considerable attention as synthetic compartments in which biochemical reactions can be carried out. Their ability to contain chemical reagents without leakage to the surrounding medium and the possibility to fuse them makes liposomes promising candidates for microdevices in which only very small quantities of chemicals are used to carry out reactions. We present an all-optical method to manipulate individual giant liposomes, typical 1-10 microns in diameter. The lipid-bilayer membrane is produced using an evaporation technique of neutral POPC lipids, and allows successful encapsulation of DNA and fluorescent dyes inside the vesicles. In order to react chemicals contained in different liposomes, we trap the vesicles in two independent optical tweezers and bring them into contact. A single pulse of UV laser light in the contact region induces their fusion thus forming a larger liposome in which the reagents from the original loiposomes mix. In the manipulation technique that we propose all mechanical contact with the liposomes is avoided and fusion is induced by a very short, 5 ns long laser pulse. Both of these aspects may allow for a better quanitification of the mixing of chemicals within the vesicles and may prove important for combinatorial chemistry using only femtoliters of reagents.

## 1-24

### Interaction of the trans-membrane fragment of bik with model membranes

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BIK is a pro-apoptotic BCL-2 family member and belongs to a subfamily known as "BH3-alone" proteins. This protein exhibits strong pro-apoptotic activity in a number of different cell types in vitro but the mechanism by which BIK promotes cell death is not yet known. BIK, apart from having a functional BH3 domain, contains a predicted characteristic C-terminal trans-membrane region. Recent studies have shown that BIK can interact with cellular membranes through its C-terminal fragment. A significant fraction of BIK, containing the predicted trans-membrane fragment, is inserted in the endoplasmic reticulum membrane, with the bulk of the protein facing the cytosol. Recent studies identify BIK as an initiator of cytochrome c release from mitochondria operating from a location at the endoplasmic reticulum. In this work we present our studies of the interaction of the C-terminal fragment of BIK with biomembrane model systems. By using infrared and fluorescence spectroscopies we have shown that this BIK fragment interacts very efficiently with membrane vesicles. This fragment presents a predominantly alpha-helical structure when inserted in the membrane which depends on phospholipid composition, being related to its binding with the membrane as observed by fluorescence spectroscopy

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## 1-25

### Study of the interaction of viscotoxins a3 and b with membrane

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Viscotoxins are small proteins which are thought to interact with biomembranes, displaying different toxic activities against a varied number of cell types, being VtA3 the most cytotoxic whereas VtB is the less potent. We have studied the interaction of VtA3 and VtB, both wild and reduced ones, with model membranes containing negatively-charged phospholipids by using infrared and fluorescence spectroscopies. Both VtA3 and VtB present a high conformational stability, and a similar conformation both in solution and when bound to membranes. In solution, the infrared spectra of the reduced proteins show an increase in bandwidth compared to the non-reduced ones indicating a greater flexibility. VtA3 and VtB bind with high affinity to membranes containing negatively-charged phospholipids and are motional restricted, their binding being dependent on phospholipid composition. Whereas non-reduced proteins maintain their structure when bound to membranes, reduced ones aggregate. Furthermore, leakage experiments show that wild proteins are able of disrupting membranes whereas reduced proteins were not. The effect of VtA3 and VtB on membranes having different phospholipid composition is diverse, affecting the cooperativity and fluidity of the membranes. Viscotoxins interact with membranes in a complex way, most likely organizing themselves at the surface inducing the appearance of defects which lead to the destabilization and disruption of the membrane bilayer.

## 1-26

### Interaction with membranes of the pretransmembrane sequence of Ebola glycoprotein

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We report the detection and characterization of a membrane-interacting domain that precedes the transmembrane anchor of GP2, the fusogenic subunit of Ebola glycoprotein. Computation of the hydrophobic-at-interface moment for this element reflects a tendency to partition into membranes adopting a helical structure. Peptides representing the GP2 pretransmembrane sequence (pre-TM) and a derived scrambled control sequence have been characterized. Only wild-type Ebola pre-TM bound the interface under equilibrium conditions adopting an alpha-helical conformation and breach the permeability barrier of membranes. The presence of the raft-type lipid sphingomyelin highly stimulated pretransmembrane capacity to desestabilize membranes. A parallel study comparing the viral sequence and melittin suggests that Ebola GP preTM sequence might use similar perturbing mechanism to damage the membrane organization of the viral envelope. The findings in this work may help in understanding the molecular mechanism by which Ebola GP2 induces membrane merging.

## 1-27

### A role for the human immunodeficiency virus gp41 pretransmembrane sequence during fusion

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The tryptophan-rich membrane-proximal region of HIV-1 gp41 glycoprotein is accesible to broadly neutralizing antibodies and, although dispensable for normal maturation, transport, and receptor binding of glycoprotein, has been shown to be essential for fusion activity. A possible role for the conserved Tryptophan residues in those processes could involve specific interactions with membrane cholesterol. Our experimental results support the notion that the pretransmembrane sequence may participate in the clustering of gp41 monomers within the HIV-1 envelope, and in bilayer architecture destabilization at the loci of fusion, a fact that prompted us to propose the pretransmembrane as a second fusion peptide present in the gp41 ectodomain. They also comfirm that only wild-type pretransmembrane sequence forms oligomeric helical structure is further stabilized upon inmersion into membranes, but self-association is only preserved in viral membrane mimetic membranes. Our data support a model according to which a trimeric helical pretransmembrane sequence couples helical bundle formation to the induction of merging by gp41.

## 1-28

### Role of $Ca^{2+}$ in the regulation of PLD from streptomyces chromofuscus

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Phospholipase D from S. chromofuscus (scPLD) is a novel enzyme non belonging to the PLD superfamily (characterized by the presence of a HKD domain) and with a dinuclear metal center similar to purple acid phosphatases (PAPs). scPLD activity is dependent on Fe3+ and, unlike PAP, it is enhanced by Ca2+ (Kd = 100 microM). A lower affinity Ca2+ binding site (Kd in the low mM range) is involved in the binding of scPLD to substrate small unilamellar vesicles. Fe3+ appears to form a coordination complex with Tyr151, as shown by visible CD and absorption spectroscopy. Resonance Raman spectroscopy shows that Cys123 is also involved in metal coordiantion. EPR spectroscopy confirmed that Mn2+ is the second metal ion as described in at least one PAP. Besides hydrolyzing aggregated and soluble phospholipids, scPLD is also active when pNPPC, bis(pNP)P and pNPP are used as substrates. The phosphodiesterase and phosphatase activities can be uncoupled through site directed mutagenesis of some of the amino acid residues (E213 and D389) hypothetically involved in the binding of cofactor metal ions. Modification of E213 also affects Ca2+ affinity to the tight site shifting Kd up to 9 mM. The role of Ca2+ in the regulation of PLD activity is under investigation by site directed mutagenesis and spectroscopic characterization of the modified proteins. Crystals of wild type scPLD have been obtained and will allow the determination of the three dimensional structure of this novel enzyme.

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## 1-29

### Antimicrobial peptides: kinetic analysis of interactions with phospholipid membranes.

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The spread of bacterial resistance to antibiotics is an increasing challenge in modern medicine. One promising solution may be the utilisation of antimicrobial peptides. These molecules are widely produced as part of non-specific host defences against infections. There is a large variety of sequences and structures among antimicrobial peptides but most share general common features. They are usually less than 50 amino acids long, have a high net positive charge and contain 50% hydrophobic residues. Many peptides adopt alpha-helical structures with a distinct hydrophobic region. The aim of this work is to investigate the mechanisms by which antimicrobial peptides interact with and disrupt biological, particularly bacterial, membranes. Large unilamellar vesicles composed of biological phospholipids were labelled with fluoresceinphosphatidylethanolamine (FPE). This fluorescent label inserts into the outer membrane leaflet and is sensitive to the electrostatic surface potential. Upon binding and insertion of peptides into the membrane the electrostatic surface potential is affected, leading to changes in the measured fluorescence. Stopped-flow mixing measurements were taken to obtain kinetic information about the early interactions of peptides with membranes. A series of antimicrobial peptides with similar sequence but distinct secondary structures were investigated using this technique. The interactions of these peptides with membranes of different composition were studied. The charge of the membrane and the secondary structure of the peptides were found to influence binding and insertion into the membrane. Antimicrobial peptides can thus be fully characterised and compared with a view to developing a set of design principles appropriate to targeting to microbes.

## 1-30

### Interaction of dystrophin rod domain and lipid membranes : affinity, electrostatic nature and involvement of tryptophan residues

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Dystrophin in the protein which is lacking in Duchenne muscular dystrophy. Its structure and function are not precisely known. We undertaken a study of the interaction of the second repeat of the central rod domain of dystrophin with lipids. To assess dystrophin rod domain membrane interactions, tryptophan residues properties of two recombinant proteins of the rod domain were examined by proton NMR and fluorescence methods in presence of membrane lipids. The first protein is a partly folded protein as inferred from NMR, tryptophan fluorescence emission intensity and the excited state lifetime. By contrast, the second longest protein is a folded compact protein. Tryptophan fluorescence quenching showed that both proteins are characterised by structural fluctuations with their tryptophan residues only slightly buried from the surface. The tryptophan fluorescence properties of the longest protein changed dramatically in presence of anionic phospholipids demonstrating a specific interaction between this protein and membrane lipids while only minor changes were observed for the non folded protein. By ultrafiltration methods, the affinity constant of the longest protein is showed to be high. The nature of the interaction is at least partially electrostatic as indicated by the dependence of the affinity upon NaCl and pH. We propose a new scheme of dystrophin molecule interactions including the interaction with plasma membrane.

## 1-31

### Binding of the c-terminal sam domain of human p73 to lipid membranes

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The alpha splice variant of p73, a homologue of the tumor suppressor p53, has close to its C-terminus a SAM motif, SAMp73, which is thought to be involved in protein-protein interactions. Here, we report, for the first time, the lipid-binding properties of this domain. Binding to lipids was assayed against zwitterionic (PC) and anionic (PA) lipids, and was studied by using different biophysical techniques (circular dichroism and fluorescence spectroscopies and differential scanning calorimetry). All these probes indicate that SAMp73 changes its structure upon binding to lipids. This binding involves surface attachment and membrane penetration. The implications in the function of p73 are discussed.

## 1-32

### Infrared spectroscopy of lipoproteins

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Lipids are transported in human blood plasma by lipoproteins consisting of a nonpolar core where triacylglycerols and cholesteryl esters are hidden surrounded by a monolayer facing the water composed of phospholipid, cholesterol and proteins, giving these lipid-rich structures water solubility. Blood plasma lipoproteins are classified on the basis of their density, which in turn is a reflection of their lipid content. The greater their lipid contents the lower their density. Three major lipoprotein classes are found in fasting human blood plasma, VLDL (very low density lipoprotein), LDL (low density lipoprotein) and HDL (high density lipoprotein). Infrared spectroscopy is nowadays a useful technique in the study of protein conformation and dynamics. The possibilities of the technique become apparent specially when applied to large proteins in turbid suspensions, as is the case with lipoproteins. Variations in lipoprotein structure have been probed by monitoring the IR amide I band. This band arises mainly from C=O stretching vibrations and is sensitive to conformational changes. External perturbations such as temperature are commonly used to obtain a deeper insight in protein structure by means of infrared spectroscopy. More recently, Noda has proposed the use of two-dimensional correlation spectroscopy (2D-IR) to increase the amount of information obtained from the infrared spectrum. In the present work we have used conventional and 2D-IR to study the temperature effect on the different lipoproteins. Bands associated with protein and lipid moieties of the synchronous and asynchronous maps have been studied to characterize the lipoproteins in different temperature ranges.

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## 1-33

**Nanoscopic arrangement of a precursor of pulmonary surfactant protein SP-B observed by scanning force microscopy (SFM)**

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Pulmonary surfactant protein SP-B is a saposin-like protein synthesized by the type II pneumocytes of lungs as a larger precursor which yields the mature protein along a proteolitic process associated with pulmonary surfactant assembly. The presence of the N-terminal propeptide in this proprotein is an indispensable requirement to ensure the correct processing and targeting of the mature SP-B in the functional surfactant complexes. This N-terminal domain of the precursor also contains an additional saposin-like motif which could protect the hydrophobic regions of the mature sequence to facilitate its accurate disposition upon assembly of the surfactant bilayers. A recombinant form of the protein precursor including the N-terminal region and the mature SP-B sequence (proSP-BdeltaC) has been produced by expression in Escherichia coli. Studies on the interaction of proSP-BdeltaC with phospholipid vesicles showed an intrinsic ability of the protein to interact with PC or PG bilayers, inducing lipid-vesicle aggregation under specific conditions. Scanning force microscopy (SFM) of proSP-BdeltaC on mica substrates or interacting with supported lipid bilayers shows discrete particles with regular sizes and shapes and an apparent diameter of 30 nm as calculated from the SFM images, compatible with the formation of multimeric arrangements by the protein. The possibility of non-covalent oligomeric patterns formed by the precursor or even the mature SP-B have to be considered to understand how this protein facilitates the arrangement of the different lipid-protein structures needed to provide a surface active film at the alveolar air-liquid interface.

## 1-34

**Model-membrane destabilizing properties of lipophilic peptides derived from an E2 domain of GBV-C / HGV**

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GB virus C (GBV-C) and hepatitis G virus (HGV) are strain variants of a recently discovered enveloped RNA virus belonging to the Flaviviridae family, which is transmitted by contaminated blood and/or blood products, intravenous drug use, form mother to child and sexually. The natural history of the GBV-C/HGV infection is at present not fully understood and its potential to cause hepatitis in humans is questionable, the mode of entry into target cells being unknown. In a previous work, the membrane-interacting properties of a potential epitope of GBV-C/HGV, located at the region (99-118) of the E2 structural protein was studied by using liposomes as model membranes. Here, to explore the lipid-lipophilic derivative peptides (palmitoyl- and miristoyl-E2) interaction, we have used two complementary experimental techniques, Langmuir-Blodget films and fluorescence spectroscopy. On one hand, kinetics of penetration on dipalmitoyl phosphatidylcholine (DPPC) monolayers indicate that both derivatives are able to incorporate in them in a similar extent. On the other hand, using fluorescence spectroscopy, the effect of peptides on the release of the encapsulated fluorophores 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) / p-xylenebis(pyridinium)bromide (DPX) was monitored by dequenching of ANTS. Parent E2(99-118) peptide did not cause leakage in large unilamellar vesicles (LUVs)-DPPC vesicles. However, Palm-E2 and Mir-E2 peptides induced leakage of vesicle contents, indicating that lipophilic peptides perturb lipid bilayers inducing permeabilization

## 1-35

**Characterization of the interaction between VHSV infectivity enhancer peptides and viral membrane using phospolipid model systems**

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We have previously reported the ability of two peptides (p5,p6) to enhanced the infectivity of VHSV, a fish rhabovirus, in susceptible cells lines ((Mas, 2002)) . Since there was no estimulation of virus infectivity when p5 or p6 were incubated with the cells rather than with VHSV, the extent of enhancements showed dependence of peptides and virus concentrations during pre-incubation, the effect was equally effective whether the cells were form carp (EPC) or from trout (RTG-2) and there was any increase of infectivity in other unrelated fish viruses, it was concluded that p5 and p6 must interact with VHSV to enhance its infectivity. In an effort to elucidate the mechanism of interaction between virus and peptides, and due to the fact that VHSV envelope is only constituted by the homotrimeric glycoprotein G ( protein G) and by a lipid bilayer proper from previous infected cell, we first investigated the possible interaction between peptides and viral membranes. We present in this work our results on phospolipid model systems by using biophysics techniques such as anisotropy, Fourier transform infrared spectroscopy (FTIR), differential scanning calorymetry (DSC) and lipid vesicle aggregation . Mas, V., L. Perez, J. A. Encinar, M. T. Pastor, A. Rocha, E. Perez-Paya, A. Ferrer-Montiel, J. M. Gonzalez Ros, Estepa, A. and Coll, J.M. (2002) Salmonid viral haemorrhagic septicaemia virus: fusion-related enhancement of virus infectivity by peptides derived from viral glycoprotein G or a combinatorial library. J Gen Virol 83, 2671-2681.

## 1-36

**Adsorption of hepatitis G synthetic peptide E2(99-118) to lipid monolayers at the air-water interface.**

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Virus-cell fusion is strongly influenced by environment pH and lipid composition of the cell's membrane. In the present study, we have determined the intrinsic surface activity of a synthetic hepatitis G peptide E2(99-118). The interactions of this peptide with monolayers of different lipidic composition, spread at the air-water interface, have been characterized. All the studies have been performed with subphases of different pH (5, 6 and 7.4). E2(99-118) showed surface activity, reac ing a saturation around subphase concentrations 1.3 mM with a pressure increase around 20 mN/m. The pH of the subphase practically did not change the surface activity values. The adsorption to lipid monolayers showed that the incorporation of the peptide was higher when anionic lipids were present such as: Dipalmitoyl phosphatidylethanolamine, Sfingomieline or Dipalmitoylphosphatidylglycerol. A high degree of interaction was also found in monolayers composed of Dipalmitoylphosphatidylcholine h and 30% Cholesterol. The pH of the subphase had a slight effect on these interactions. In general the higher incorporation of peptide into the monolayers was found for pH 6. All these data suggest that the interaction of E2(99-118) with membrane lipids is maximum at pH 6 and that the presence of cholesterol and negative charged lipids in DPPC monolayers increase these interactions.

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## 1-37

**Natural emulsifiers at fluid-liquid interfaces**

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The characterization of biological interfaces often requires modelling of interfacial phenomena encountered in these systems by the concepts of surface science in order to analyse them in terms of basic molecular features. The monolayer technique allows a characterization of the system on a molecular scale. Many biological processes rely on the diverse interactions between molecules and hence the study of monolayers of different natural emulsifiers, like lipids or proteins, becomes a useful tool in order to predict or control a wide range of biological processes. In this research paper a comparative study between natural emulsifiers at both, air-water and oil-water interfaces is presented in terms of their behaviour in monolayers. For this purpose a novel langmuir type pendant drop film balance has been used that offers several advantages over conventional balances such as the small volume needed and rapid rates of surface area changes. The different behaviour is analysed in terms of interactions between molecules and between molecules and interface.

## 1-38

**Plastocyanin interaction with lipid membranes and membrane bound proteins**

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Plastocyanin is a mobile electron carrier that transports the electrons between the two membrane-embedded photosynthetic complexes cytochrome b/f and Photosystem I presumably over long distances (up to 500 nm) during the photosynthesis. The electron transport takes place in the thylacoid lumen with a transverse dimension of about 10 nm. Plastocyanin is a small blue copper protein of 10.5 kDa containing one Cu atom per protein, which alternates between Cu(I) and Cu(II) oxidation states.
The purpose of the project is to study the redox and pH dependent interaction of plastocyanin with photosystem I, with cytochrome b/f as well as with the lipid part of the membrane using PAC (perturbed angular correlation) spectroscopy.
It has been shown that the interaction of plastocyanin with photosystem I is strongly dependent on the redox state of the protein [1]. The binding of plastocyanin to the lipids has been shown to depend on the pH and redox state [2]. The structural background as well as the consequences for the long-range electron transport is being investigated.

## 1-39

**Effect of lipopolysaccharide on the properties of the N-terminal segment of pulmonary surfactant protein SP-C to interact and perturb phospholipid membranes**

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Pulmonary surfactant is a lipid-protein complex which main function is to modulate the surface tension at the alveolar air-liquid interface of lungs during the breathing cycles, to facilitate respiratory mechanics. This material plays also important roles in innate defence mechanisms at the respiratory epithelium. SP-C, one of the specific surfactant proteins, is a 35 amino acid polypeptide containing an hydrophobic transmembrane □-helix and a 12-residue cationic N-terminal segment with two palmitoylated cysteines.
Different peptides with sequence corresponding to the N-terminal segment of SP-C have been synthesised to study structure and lipid-protein interactions of this region of the protein. The N-terminal peptides were water-soluble but spontaneously interacted with zwitterionic (PC) and anionic (PG) phospholipid membranes, inducing pronounced perturbations on their structure as observed by peptide-induced aggregation and content-leakage of phospholipid vesicles. Some of the N-terminal SP-C peptides, as well as the whole native SP-C, were able to bind lipopolysaccharide (LPS).
The effect of the presence of LPS in the membranes has been examined with regard to the membrane-perturbing properties of the N-terminal segment of SP-C. The results will be discussed in terms of potential roles of the protein on the interaction of pulmonary surfactant with pathogens.

## 1-40

**Dsc study of the interactions between two hybrid peptides and DMPC and DMPG liposomes**

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The growing resistance of bacteria against conventional antibiotics has led to an intense research for new types of antibiotics. On recent years, much research has been done on a new class of these drugs, the antibiotic peptides, as the defensive role of a variety of naturally occurring antibiotic peptides is recognized. In the present work we did synthesize two cecropin A - mellitin hybrids, namely the CA(1-8)M(1-18) and the CA(1-8)M(2-9), by Fmoc/$^{t}$Bu solid phase strategies. The peptides were purified by reverse phase liquid chromatography and characterized by analytical HPLC, amino acid analysis and MALDI-TOF mass spectrometry. LUV's from DMPC and DMPG were used as model membranes, and were prepared in HEPES buffer, pH 7.4. A MicroDSC (Setaram MicroDSC III) was used to follow changes in the phase transitions of liposomes, as a function of the peptide/lipid ratio for both hybrids. The observed changes in the thermal profile – transition temperature, enthalpy change associated with the transition and change of the profile itself, are discussed in terms of the type of interaction between the peptides and the liposomes. A very different behaviour is observed both depending on the peptide and on the lipid used.

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## 1-41

**Interaction of moxifloxacin with large unilamellar liposomes: partition studies reveal the importance of charge interactions.**

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Fluoroquinolones are antimicrobial agents widely used in the treatment of a large number of infections. Their activity results from the inhibition of bacterial DNA gyrase activity, an essential enzyme required for DNA replication, transcription, repair and recombination. Fluoroquinolones use a transmembranar protein (porin) to pass through the outer membrane of the gram negative bacterials, but the exact mechanism and the previous interactions between the drug and the lipidic composition of the outer-membrane, wish may influence the opening of the channel, remains unknown. Thus the study of the mechanism of action of the fluoroquinolones falls in part in the domain of membranology, particularly in the investigation of the nature of the interaction between these drugs and different phospholipidic compositions of the membrane.In this study, the partition equilibrium of moxifloxacin was quantified by the determination of its partition coefficient, Kp, using liposomes as bacterial cell membrane models. Large unilamellar liposomes of DMPC (dimyristoyl-L-alfa-phosphatidylcholine) and DMPG (dimyristoyl-L-alfa-phosphatidyl-glycerol) were used. The liposome/aqueous phase partition coefficients were determined at physiological conditions, by derivative spectrophotometry and fluorimetry Moxifloxacin show stronger interactions with the negatively charged phospholipids (DMPG), while the interactions with DMPC, a zwitterionic phospholipid, were much weaker. In view of these results, the mechanism by which moxifloxacin permeates through the phospholipid bilayer must include an electrostatic adsorption at the interface region and this association must be the first step that governs the mechanism of interaction of these drugs with bacterial natural membranes. This behavior is compared with that observed for other fluoroquinolones.

## 1-42

**Secondary structure of amphipathic model peptides at the air/water interface and their binding to lipid monolayers as studied by IRRAS.**

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Amphipathic peptides play an important role in the defense systems of several species. For instance, melittin from bee venom and magainins from the skin of amphibians show a high cell-lytic activity. Infrared reflection-absorption spectroscopy (IRRAS) was used to study the behavior of amphiphilic molecules at interfaces. A model peptide with the sequence KLALKLALKALKAALKLA-$NH_2$ (KLAL) and several analogue peptides mimick the behavior of natural peptides. KLAL is known to form a random coil in salt-containing buffer solution at low peptide concentrations, becoming ß-sheeted above a certain threshold concentration. At the air-water surface, however, even at concentrations much lower than the bulk threshold concentration, KLAL shows a ß-sheet structure. Spread monolayers of KLAL tend to be α-helical at high area per molecule. Compression of the monolayer leads to aggregation on the surface resulting in ß-sheet structures. KLAL binds strongly to anionic POPG monolayers, but shows only a low affinity to the neutral phospholipid POPC, It is α-helical in its bound form. Expansion of the lipid monolayers leads to a conversion of the α-helical form to a ß-sheet structure. For POPG films, consecutive compression converts the ß-sheet structure back to an α-helix. The results indicate that the binding of KLAL to lipid monolayers is driven mainly by electrostatic forces if the monolayer contains anionic phospholipid. The assumption that a peptide´s secondary structure at the air/water interface or in its bound form to bilayers is the same as in solution does not apply to KLAL and its related analogues.

## 1-43

**Interactions of Poly(L-lysines) with Negatively Charged Membranes.**

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Electrostatic interactions play an important role in protein-binding to membranes. We investigated complexes of DPPG-containing membranes with poly(L-lysines) (PLL) as simple model systems. The goal of the work was to study the effect of PLL adsorption on the phase behaviour and structure of the membrane as well as the secondary structure of the adsorbed peptide.
Differential-Scanning-Calorimetry (DSC) experiments revealed a rise of the main-transition-temperature of bilayers containing DPPG upon binding of poly(L-lysine) (PLL). Longer PLLs (200 – 900 residues) show a larger effect than shorter ones (10-100 residues). Using lipid mixtures with DPPC and excess concentrations of either the lipid or the polypeptide component, we observed domain formation within the membranes and up to three different transition peaks.
FT-IR spectroscopic experiments show that the binding of PLL induces a higher order in the hydrophobic part of the membrane in both phases. PLL binds in an α-helical conformation to the charged DPPG membranes, while its conformation in the bulk at pH 7 is random coil. The amount of bound α-helical peptide increases with chain length and decreases with the temperature.
The major results are that the main transition temperature of DPPG and DPPG/DPPC membranes are directly affected by the amount of α-helical bound peptide in the gel-state and that the phase transition can induce an α-helix to random coil transition of bound PLL.

## 1-44

**Mechanistic understanding of polymyxin b contact formation using a monolayer-vesicle system.**

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Polymyxins (PxB, PxE) are peptide antibiotics very effective against Gram-negatives, including *Stenotrophomonas maltophilia* which is resistant to most broad-spectrum antimicrobial agents. PxB is also the most effective agent for the treatment of sepsis. Despite its activity and the fact that no genetic resistances to PxB have been described, its clinical use is limited on account of its toxicity. In order to develop new antibiotics that possess the therapeutic properties of polymyxin but not its harmful side effects, it is necessary to understand its mechanism of action on the membrane. Having demonstrated that PxB forms vesicle-vesicle contacts that are stable and support a fast and selective exchange of phospholipids, we now use an asymmetrical monolayer-vesicle system to get mechanistic understanding of contact formation and lipid transfer. Polymyxins insert efficiently into monolayers, with a specificity for anionic phospholipids. Binding stoichiometries at a given lateral pressure are calculated for different lipids, including the lipid matrix of the membranes of *Escherichia coli*. Inserted PxB and PxE, but not the deacilated nonapeptide, promote contact formation with vesicles in the subphase, with lipid flow from the vesicles to the monolayer. Lipid transfer depends on the composition and the difference in lateral pressure in the two apposed interfaces. Mixed lipid/peptide monolayers are transferred onto solid surfaces and investigated by atomic force microscopy (AFM). The results lead to new suggestions on the involvement of intermembrane contact formation in antimicrobial action.

## *Posters*

*- Lipid-protein interactions -*

## 1-45

**Determination of the interaction between a synthetic peptide antibiotic and model membranes.**

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Due to the fact that bacteria develop an increasing resistance against common antibiotics it is necessary that new antibiotics not working on the classical principle of receptor induced reaction/destruction of bacteria have to be developed. A promising candidate for the development of an antibiotic is the peptide used in this work called NK-2. It was reported that the peptide NK-2 is highly active against bacteria but does not exhibit cytotoxicity against human cells. A direct action without a receptor on the membranes of the target cells is observed.
As model membrane systems different phospholipids mimicking the cell membranes of bacteria or erythrocytes were used. The X-ray small angle scattering technique was used to determine the influence of NK-2 on the repeat distance of the lipid bilayer and the phase transition temperature of the system. The data from this technique show that the phase transition temperature of PE is decreased after an addition of NK-2 which means that the lipid membrane becomes more fluid. In combination with the grazing incidence X-ray diffraction technique, which can give an answer about the orientation of the phospholipid on the liquid interface, the mechanism of destruction of the model membranes by the peptide NK-2 can be deduced.

## 1-46

**Mechanical Coupling via the Membrane Fusion SNARE Protein Syntaxin-1A: A Molecular Dynamics Study**

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SNARE trans-complexes between membranes likely promote membrane fusion. For the t-SNARE syntaxin-1A involved in synaptic transmission, the secondary structure and bending stiffness of the 5-residue juxtamembrane linker are assumed to determine the required mechanical energy transfer from the cytosolic core complex to the membrane. These properties have here been studied by molecular dynamics and annealing simulations for the wild type and a C-terminal-prolongated mutant within a neutral and an acidic bilayer, suggesting linker stiffnesses above 1.7, but below 50 x cal $mol^{-1}$ $deg^{-2}$. The transmembrane helix was found to be tilted by 15° and tightly anchored within the membrane with a stiffness of 4-5 kcal $mol^{-1}$ $Å^{-2}$. The linker turned out to be marginally helical and strongly influenced by its lipid environment. Charged lipids increased the helicity and H3 helix tilt stiffness. For the wild type, the linker was seen embedded deeply within the polar region of the bilayer, whereas the prolongation shifted the linker outwards. This reduced its helicity and increased its average tilt, thereby presumably reducing fusion efficiency. Our results suggest that partially unstructured linkers provide considerable mechanical coupling; the energy transduced cooperatively by the linkers in a native fusion event is thus estimated to be 3-8 kcal/mol, implying a two to five orders of magnitude fusion rate increase.

## *Posters*

*- Theoretical biophysics -*

## 2-1

### The membrane potential of fish embryos and the mathematical model of its generation

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Synchronous cell divisions after fertilization of oocytes are accompanied by periodic fluctuations of membrane potential and other bioelectrical parameters on the background of monotonous changes of their basic level. In this work the temporary changes of membrane potential and of the activity of Na/K-ATPase of loach embryo cells during their synchronous divisions of 7 hours of development were investigated. Membrane potential was registered with microelectrode technique. The variations of Na/K-ATPase activity were determined in the absence and presence of ouabain, respectively. The obtained results show that during synchronous cell division of blastomeres the membrane potential increases in the same order with the activity of Na/K-ATPase (its electrogenic component), and with the changes of intracellular concentration of ions (its diffusional component). An additional dynamic component of potential appears due to the unbalance between flows of cations through the membrane.
The models for temporary changes of electrophysiological characteristics of loach embryo cells that describe the aperiodic trend and the oscillations of membrane conductance were developed. Their combination results in complete model for dynamics of these membrane-associated processes. It provides mathematical description for membrane potential, voltage-dependent conductance, ion currents and intracellular concentrations of potassium and sodium ions. It accounts also the interdependence of sodium and potassium conductance, which determines the occurrence of periodic oscillations. Thus the complete quantitative picture of temporary changes of all bioelectrical parameters during 7 hours of embryo development can be simulated, and appearance of periodic oscillations of electrophysiological characteristics can be described in a consistent manner.

## 2-2

### Interpretation of DPPC structure by Raman spectroscopy and ab initio calculations

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The well-established use of Raman spectroscopy to examine the structure of bio- membranes is extended through a spectrum-structure relationship by means of Raman intensity ab initio calculations. In this paper FT-Raman spectroscopy was used to evaluate the organisation and thermal transition properties of DPPC liposomes. Raman spectral parameters, such as band frequencies, intensities, widths and shapes were analysed. Analysis of those parameters yield information on conformer population, reorientational fluctuations, hydrogen bonding, packing and phase transformations of the lipid bilayers. In addition, in this paper we present quantum mechanically (Hartree-Fock, Density Functional Theory) calculated Raman frequencies and intensities which will be interpreted along with the experimentally observed frequency shifts and intensities. All calculations are performed with the Gaussian98 or DGauss 5.0 suite of programs on a CRAY supercomputer (SV1 or T3E).

## 2-3

### Mechanism of oxidative damage to DNA

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We study throug computer simulation the oxidative damage to DNA, which is a common cause of mutagenesis, cancer and cell death. Since guanine (G) has the lowest oxidation potential among nucleic acid bases and holes can travel long distances along the DNA strands, it is believed that guanine radical cation (G+.) is the first step in the oxidation process. Much less clear are the subsequent steps. The aim of this study is to elucidate the complex chemical reactions that take place after the formation of G+. and to assess whether or not there is an energetic funnel that guides the oxidation process towards a single product. Achieving this goal requires taking into account the complex environment in which the oxidation process takes place. To this effect we use state-of-the art molecular dynamics simulations, taking advantage of large computational resources and novel computational methods. In particular, we have used an efficient and accurate QM/MM method and the novel metadynamics approach, which allows long time scale phenomena to be studied. We conclude that DNA is engineered in such a way that at the end of the oxidation process a single product is generated, namely 8-oxoguanine.

## 2-4

### Modeling of ion transport in pH dependent fixed charge membranes with narrow pores

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The ionic transport through pH dependent fixed charge membranes with narrow pores constitutes a problem of biophysical interest. We present here a simplified model based on the Nernst-Planck flux equations under the Goldman constant field approximation for the different species transported through the membrane together with a Langmuir type isotherm for the possible adsorption of divalent ions onto the membrane surface. Special attention is paid to the local dissociation equilibria of the fixed charge groups within the membrane. The model allows for the calculation of the fluxes of all the ionic species involved as well as the estimation of the reversal potential and the conductivity of the membrane. Comparison of the theoretical results with experimental data obtained from polyethylene terephthalate and polycarbonate track-etched membranes having narrow pores show that the model can explain qualitatively the transport of small ions through pH dependent fixed charge membranes with pore radii higher than 1 nm approximately, but it fails when attempting to describe the transport of large ions through smaller pores

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*- Theoretical biophysics -*

## 2-5

**Topology and biopolymers: how to tame DNA-knots and other animals**

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In bacteria, the DNA usually occurs in ring form. However, this ring may actually come knotted. Topoisomerases, specialised enzymes, reduce the knottedness of the DNA under ATP-energy consumption, to enable replication. The knot detection through the topoisomerases is very specific, and to understand the possibility to determine the knottedness from local information for longer DNA, we use statistical mechanical tools to determine whether in a flexible polymer a knot will, at equilibrium, be tight or not. Exact results are presented for flat knots, and 3D knots are treated within a phenomenological model. Some predictions of the influence of persistence length and dense effects are included. Our calculations are corroborated by Monte Carlo analyses. Further applications of our approach concern the long-distance looping in DNA and the required minimum binding energy to compensate the entropy loss upon looping; and the denaturation transition of dsDNA, including its flucutations at the transition.

## 2-6

**A kinetic proofreading mechanism for protein sorting and vesicle formation in the golgi apparatus**

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Coated vesicles play a central role in maintaining the steady state distributions of resident proteins in the exocytic pathway. The distribution of glycosylation enzymes in the Golgi apparatus, for example, is a consequence of continuous recycling through coatomer protein I (COPI) coated vesicles. The sorting of cargo enzymes into COPI vesicles is an active process requiring GTP hydrolysis by the small GTPase ARF-1. In its GTP state, ARF-1 binds to Golgi membranes and subsequently, recruits coatomer. This coat-complex then polymerizes and deforms the membrane thereby enforcing budding and COPI-vesicle formation. The hydrolysis of GTP by ARF-1 is on the other hand also known to release coatomer from the membrane. At first glance, such a release would seem counterproductive to vesicle formation. We explain here how in fact, this promotes active sorting. Exploiting the recently reported down-modulation of ARFGAP1 activity by resident proteins, an efficient kinetic proofreading mechanism is can be formulated that allows for selection of proper enzymatic cargo, coat polymerization, and vesicle formation. It supports sorting of resident proteins at distinct places in the pathway, even when these are present in minor amounts.

## 2-7

**The kinetics of double enzymatic cycling assays**

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A mathematical description has been made of an enzyme amplification mechanism involving the coupling of two substrate cycles. In this amplification system one of the non-cycling products of a first substrate cycle acts as a trigger molecule that feeds a second substrate cycle. This procedure has been applied to measure nanomolar levels of NADP-NADPH and ADP-ATP, although always in discontinuous assays. This fact, together with the absence of time-based equations that would make it possible to follow the process in a continuous form, led us to solve the corresponding set of differential equations. Thus time-concentration equations describing the evolution of species involved in the system have been obtained for different experimental conditions. The model is applicable to the amplified analytical determination of low levels of a metabolite or an enzyme activity, and its amplification capacity as well as the simplicity of determining kinetic parameters enable it to be employed in enzyme immunoassays to increase the magnitude of the measured response.

## 2-8

**Expressing biochemical network models in SBML**

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The Systems Biology Markup Language (SBML) is an XML-based standard language for storing and sharing models of biochemical reaction networks. A consortium of developers of biochemical systems simulation and analysis software convenes twice a year to specify the language, and to delineate directions for further development. A smaller executive sub-committee then converts the recommendations of the consortium into a coherent language definition, and also develops tools that facilitate its integration. A dozen or so simulation and analysis packages already support SBML, or are in the process of integrating it. Once models have been entered into one of these packages and expressed in SBML, they can then be exchanged between all SBML compliant tools without the need to enter them again in different formats. A selection of models that have been expressed in SBML are available from a repository on the SBML website (http://sbw-sbml.org/index.html). The models in the repository vary from simple single step schemes to elaborate systems composed of hundreds of reactions distributed over many compartments, and describe signalling cascades, mitotic and circadian oscillators, metabolic pathways, and a variety of other processes. The SBML language definition, tools that facilitate SBML integration into existing or new software, many SBML compliant packages, and all models in the repository, are freely available for downloading from the website, and modellers are encouraged to submit their own models to the SBML repository.

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## 2-9

### Thermodynamic model of skeletal muscle contraction

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Muscle, as the complex biophysical system, attracts attention of many researchers. In particular, the new experimental data on the study of muscle contraction [1-3] are not coordinated with so-called theory of slip and the actual problem is to explain the observed experimental facts within the framework of the correct biophysical model. In the present paper the new model of skeletal muscle contraction, which is based on the general thermodynamics ideas of muscle shortening is proposed. In particular, we have considered a sarcomere, in that appear movements with continuous or quasi-continuous set of impulses, due to which sarcomere functions. The statistical sum of such biosystem, its free energy and equation of state for sarcomere (the "stress - length" dependence) were analytically obtained. The motion equation of skeletal muscle was found, one part of which was rigid, while to another - the free one - active and passive external forces were applied. It was found the limited stress value of the beginning of isotonic muscle contraction and the value of maximum possible muscular strain. Finally, the adjusted Hill equation (the "speed of contraction - stress" dependence) was obtained and analyzed in detail. The obtained theoretical results are in a good agreement with experimental ones [1-3]. References 1. Wahr P.A. and Metzger J.M. J.Physiol. 85, 76 (1998). 2. Edman K.A.P. J.Physiol. 519, 515 (1999). 3. N.S. Miroshnichenko, I.A. Zaloilo and Yu.I. Prylutskyy. In "Modern Problems of Biophysics", Donetsk, Lebid?, 2001, p. 35-49.

## 2-10

### Molecular mechanics simulations of proteins at constant pH

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An algorithm for simulation of protein dynamics at constant pH, under development, is based on a combination of a theoretical method for prediction of protonation equilibria of its titratable residues with one of the three simulation methods implemented in the Molecular Dynamics program, CHARMM: simple Langevin dynamics, Molecular Dynamics with either implicit or explicit solvent. The idea behind our approach is simple. The whole simulated protein trajectory is divided into smaller parts. Each sub-period of the simulation is preceeded by an evaluation of protonation probabilities of titratable groups in the starting structure at a selected pH. With these probabilities, the next step is to decide what is the actual protonation state assumed in the simulation sub-period to follow, by a Monte Carlo type approach. The procedure can be used for predicting pKas of titratable groups, and a comparison with experimental pKas serves as a test of reliability. However, we want to use this methodology for an investigation of pH-structure relationships in proteins, relaxation of structures under a pH-jump, changes in protonation patterns during protein-ligand association, etc. Results obtained with Langevin dynamics are very promissing. We are currently testing Molecular Dynamics variants of this procedure using the ovomucoid third domain, and also several polypeptides, as model systems, all with pKas of their titratable residues known from NMR titration experiments.

## 2-11

### Theoretical and experimental study of the rec-a homology search mechanism

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Homologous recombination plays a key role in generating genetic diversity and repairing damaged DNA. It consists in the exchange of DNA fragments with similar sequences. In this respect, the RecA protein system, which has numerous analogs among all organisms, provides a valuable paradigm for the study of homology recognition and strand exchange. Once RecA has coated a single-stranded DNA, structuring it as a helix overextended 1.5 times in comparison with the canonical double-helix, this nucleofilament must find a homologous naked duplex DNA partner. This can be envisioned as an extended sequence-specific binding entity searching for a target amid non-specific sequences. We focus on the complex and efficient homology search performed via RecA. To understand the effects of the physico-chemical structure of the polymeric molecules to be recombined, we propose a theoretical modelling approach, taking into account two important features of the search : multiple simultaneous contacts between searching and target molecules, and longitudinal breathing of duplex DNA. Attractive non-specific interactions between nucleofilaments and naked DNA are indeed believed to be responsible for facilitated diffusion based on intersegment transfer. Then, once a fragment of nucleofilament has established a loose contact with its homologous counterpart, the duplex DNA has to locally overstretch before perfect base alignment and stabilization of the joint are achieved. Fluctuations between the usual B state of DNA and a metastable stretched state should therefore be essential in the process. Single molecule experiments are under development in order to monitor homology search in real-time and thus test the model.

## 2-12

### Factors influencing the pore formation rate in the cell membrane during electroporation

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The following factors which influence the pore formation rate after the appearance of pores in the cell membrane are discussed: (i) decrease of the membrane resistance in that part of the membrane where pores are formed, (ii) equilibration of the concentration of the potential determining ions in the cell and extracellular medium, (iii) decrease of the local transmembrane potential owing to the influence of the "spreading resistance" and (iv) change of the intracellular potential (redistribution of the total voltage on the cell $\Delta\Phi_{cell}=3E_0a$).

It has been analyzed when these factors can change the pore formation rate to a considerable extent. It has been shown that the change of the rate $k_f$ owing to all factors can be ignored as long as the number and size of pores are small ($n < 100$, $r < 1$ nm).

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## 2-13

### Structure and dynamics of RNA motifs: non-watson-crick base pairing, ordered hydration and cation binding

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Thorough insight into the structure and dynamics of various biologically important RNA motifs (bacterial and chloroplast 5S rRNA Loop E, BWYV frame-shifting pseudoknot) has been obtained by state-of-the-art molecular dynamics simulations with explicit representation of solvent and counterions. The simulations provide unique information about the role of non-Watson-Crick base pairing in these RNA molecules. One of the major outcomes of our studies is the observation of a number of unusual long-residency hydrations sites. Highly ordered hydration sites exhibit almost 100% site occupancies. Some of them are dynamic, with fast water exchange on the usual time-scale of 0.1-0.3 ns, while the other hydration sites show unusually long residency times on the order of nanoseconds. These long-residency hydration sites have been found in hydration pockets of studied RNA molecules, as the phosphate cluster of the frame-shifting pseudoknot, the A-stack motif of Loop E, but have been also found in DNA zipper motif and DNA-DAPI complex. In addition, simulations provide an important insight into the cation binding. Unique sites for cation binding have been found in the deep grove of Loop E. Binding of Mg2+ rigidifies Loop E and stabilizes its major groove at an intermediate width. In the absence of Mg2+, the Loop E motifs show an unprecedented degree of inner-shell binding of monovalent cations and a wide range of deep groove widths, depending on the base sequence and the counterion distribution. Ordered hydration and cation-binding sites represent an integral part of the solute molecule and are intimately connected with local conformational variations of the solute molecule.

## 2-14

### Enzymes in nonaqueous solvents: protein structure, dynamics and enantioselectivity

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Biocatalysis in organic solvents has been successfully used in selective and stereospecific catalysis of organic compounds, with several potential advantages[1]. It is known that in nonaqueous media, the presence of water is essential for catalysis[2] and counter-ion neutralisation has also an important role[3]. The molecular reasons for their behaviour are largely unknown. With simulation studies we have addressed the problem of protein structure and dynamics in nonaqueous solvents, using ubiquitin and cutinase[4]. These studies showed that protein structure and dynamical behaviour in hexane where more similar to the ones found in water, only in a narrow range of hydration (5-10% (w/w)). Lower water contents generate a too rigid structure with significant deviations from the aqueous conformation, while higher water content lead to unfolding. This can explain the characteristic bell-shaped behaviour of catalysis under different hydration conditions. Enzyme activity of proteins can be studied using theoretical methods. In serine proteases, the differential binding of tetrahedral intermediates can be used as an estimate of catalytic rates[5]. Using this hypothesis, we studied cutinase enantioselectivity with the Free Energy Perturbation (FEP) approach. The results can be successfully correlated with experimental data[6] and reveal the underlining factors responsible for cutinase enantioselectivity. 1. Klibanov, A.M., 2001 Nature, 409: 241-245. 2. Zaks, A. et al., 1988 PNAS, 83: 3192-3196. 3. Halling, P.J., 2000 Curr. Opin. Chem. Biol., 4:74-80. 4. Soares, C.M., et al., 2002 Biophys J. Accepted. 5. Warshel, A., et al., 1989 Biochemistry, 28: 3629-3637. 6. Fontes, N.M., et al., 1998 I&EC Research, 37: 3189-3194.

## 2-15

### Stretching of a polymer below the theta point

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The unfolding of a polymer below the theta point when pulled by an external force is studied both in d=2 on the lattice and in d=3 off lattice. At T=0 and for finite length chains, it is found that the globule unfolds via multiple steps, corresponding to transitions between different minima, in both cases. Remarkably, the succession of these minima explains some recent experimental data on polymer stretching. Moreover, we found that in d=3 one of these intermediates is a regular helix, one of the basic building blocks of the proteins. In the infinite length limit, these steps have a qualitative effect only in d=2. The phase diagram in d=2 is determined via the transfer matrix technique. To rationalize these results, energy-entropy and renormalization group arguments are given.

## 2-16

### Bundling and desorption of semiflexible filaments

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Bundling and desorption transitions of semiflexible filaments, such as actin filaments, microtubules, or DNA, are discussed. For the bundling of two filaments and the desorption of a single filament from a planar surface we give a complete classification of the bundling transitions as obtained by transfer matrix methods and scaling arguments. The interaction potentials can depend both on the polymer/polymer (or polymer/surface) separation and on the orientation of the polymers. The cooperative nature of the bundling transition for three or more filaments is studied by Monte-Carlo simulations and scaling arguments. Finally we discuss force-induced desorption of single semiflexible filaments from an adhesive surface to obtain the complete phase diagram as function of force and adhesion strength. Our results are applicable to biopolymers or polyelectrolytes with large persistence lengths such as actin filaments, microtubules, or DNA.

## *Posters*
*- Theoretical biophysics -*

## 2-17

**A molecular modelling study of the structural properties of αB-crystallin and αA-crystallin, two members of the sHSP family.**

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Among the small heat shock protein family (sHSP), the αB-crystallin is expressed in a number of tissues (lens, skeletal and cardiac muscle) while its homologous, the αA-crystallin, is expressed only in lens. Both proteins possess chaperon-like function. Oligomerization is a prerequisite for its chaperone function. The sHSP family is characterized by a well conserved structural domain in the C-terminal part: the α–crystallin domain.
A missense mutation in the αB-crystallin, R120G, is genetically linked to a desmin-related myopathy and cataracts, due to the loss of its chaperon activity. This arginine residue is almost conserved for all the members of the sHSP family. For αA-crystallin, the equivalent mutation R116C leads to the development of cataracts. The functional changes are associated to the formation of non-specific aggregates. Despite a strong functional and structural similarity, many differences exist between the two proteins, particularly, concerning the features of the assemblies forming the chaperone complex.
In order to gain insights of the two protein properties, we have undertaken a molecular modelling study, for the two wild-type proteins and their respective mutant. Their structure has been established by homology modelling from Xray-structure of related proteins Hsp16.5 and Hsp16.9. Molecular dynamics simulations were performed for the monomeric structures. Different structural properties were systematically examined. Docking procedures have been applied in order to determine the zones involved in the formation of the complex. The ensemble of properties deduced from these calculations for both molecules and their mutants is discussed in the light of the experimental biological results.

## 2-18

**Determining the middle of Escherichia coli**

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The rod-like bacterium Escherichia coli divides by growing a wall perpendicular to its long axis. This septum is located in the middle of the bacterium. Positioning the septum involves pole-to-pole oscillations of the proteins MinD and MinE. Experiments in vitro show, that the ATPase MinD attaches to the cytoplasmic membrane when bound to ATP. On the membrane, it aggregates into filamentous structures. The protein MinE associates with membrane-bound MinD. It induces hydrolyzation of ATP by MinD and thereby drives MinD off the membrane. In the absence of MinD, however, MinE is found in the cytosol. It is shown here, that these features of the dynamics of MinD and MinE suffice in order to generate the experimentally observed oscillations. To this end a generic description of the protein dynamics is presented. Linear stability analysis of the stationary homogeneous state reveals, that the oscillations are due to a Turing instability. Numerical integration of the dynamic equations show, that the essential features of the oscillations are captured. Based on the theoretical analysis, a mechanism for the initiation of cell division is proposed. Finally, similarities and differences to alternative descriptions of the protein dynamics are discussed.

## 2-19

**Computational engineering of protein-protein interfaces for structure-based drug design : from detecting binding sites and identifying hot spots on pr**

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Protein binding sites are generally not only structurally flexible, but also functionally adaptive, with a diverse range of protein systems capable of binding with high affinity to ligands different from their natural binding partners. in composition, size, and shape. I will discuss novel approaches for computational mapping of protein-protein surfaces and detecting binding sites and energetic hot spots at intermolecular interfaces. In a first study, we describe the application of a hierarchical computational approach to identify the engineered binding site and hot spots at the remodeled intermolecular interface between human growth hormone and the extracellular domain of its receptor. In the second example, we study the relationship between conformational flexibility and convergent evolution in recognition with the hinge region of the constant fragment (Fc) of human immunoglobulin G (Ig) protein, which represents the consensus binding site for natural proteins and synthetic peptides. Finally, we present the results of the computational design approaches complementing 'orthogonal' chemical genetic strategy for designing novel inhibitor scaffolds and engineering specificity in Src tyrosine kinases

## 2-20

**Complexation of Cu(I) by the metallochaperone Atx1**

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Chelation of metal ions (Cu(I), Co(II), Cd(II), Pb(II)) in Prokaryotes and Eukaryotes involves proteins rich in thiolate groups with the metal-binding consensus sequence CxxC. These trace metals are essential to cell functions but highly toxic in excess. We study the mechanism of selectivity for Cu(I) of the copper chaperone Atx1 from the yeast Saccharomyces cerevisae, by two complementary approaches: a structural one (protein conformation, metal coordination sphere and characteristics of the metal-protein binding) and a thermodynamic one (affinity). The molecular mechanism and the reactivity of the CxxC site towards the Cu(I) ion have been investigated, both structurally and energetically, by quantum chemistry on the model (CH3S-)2Cu+. The CHARMM molecular mechanics force field has been parameterized to reproduce the ab-initio potentials obtained from quantum mechanics calculations. Cu-S interactions are introduced as harmonic "bonded" terms (bond stretching and angle bending) while Cu-water interactions are treated through a non-bonded Lennard-Jones type potential. The affinity of either Atx1 or Atx1 biomimetic peptides for several metals, or the affinity of some mutants of the protein for a given ion will then be calculated and compared to experiments. The optimized force field has already been used for preliminary molecular dynamics studies of the stability of the metal-protein or metal-peptide systems.

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## 2-21

### Stochasticity in intracellular calcium dynamics

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We investigate the behaviour of calcium in the cytosol by stochastic simulations. Our approach allows to start modelling on the level of a single subunit of the Inositol-1,4,5-trisphosphate (IP3) receptor but still allows to describe structures on large length scales. The model is based on the DeYoung-Keizer model and exploits an adiabatic elimination of the dynamics of the calcium concentration on small length scales. The simulations show oscillations with periods between 17s and 2 min with a deterministic and a stochastic part. The latter can be explained by a nucleation process. In the deterministic limit of the model the oscillations disappear and a high activity stationary state emerges. Thus, fluctuations are vital for the formation of spatial and temporal structures in intracellular calcium dynamics.

## 2-22

### Calcium profiles of single channel clusters at the membrane of the endoplasmic reticulum

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We study the calcium concentration profiles that are generated by Ca2+ release from the endoplasmic reticulum through clusters of IP3 receptor channels. The release is simulated in geometries mimicking the situation in the cell. We find that peak values close to the channel cluster exceed typically measured bulk averages by at least one order of magnitude. Simulation results enter a stability analysis of models of calcium induced calcium release. Stability and behaviour of the models changes essentially when realistic concentration values are used instead of averaged bulk values.

## 2-23

### Hydro: a suite of computer programs for the calculation of solution properties of rigid macromolecules and bioparticles

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HYDRO is a collection of computer for the calculation of hydrodynamic coefficients and other solution properties of macromolecules or other rigid particles. The programs embody the hydrodynamic theory of rigid particles represented by arrays or shells of beads, and also construct the bead/shell models for various cases. HYDROPRO works with structures with atomic resolution, taken from PDB file, and HYDRONMR gives, in addition, the NMR correlation time and residue-specific relaxation times, T1 and T2. HYDROMIC performs a similar task, for macromolecular structures determined by cryo-electron microscopy, starting a spider structural file. HYDROSUB is for structures composed subunits whose structure is represented in a simplified manner, as spheres, ellipsoids or cylinders. HYDROPIX, is for a general, arbitrary structure that can be programmed by the user. We have other ancillary programs that employ the output of those mentioned above. SOLPRO evaluates other solution properties, like scattering form factors and the time decay of birefringence, or fluorescence anisotropy, and evaluates shape-related, size-independent quantities parameters, like the Einstein, Perrin and Flory parameters. Finally BROWNRIG simulates and analyzes Brownian dynamics trajectories of rigid particles, consider the presence of external agents like electric fields or hard obstacles.

## 2-24

### Why do small globular proteins fold co-operatively?

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The question of how a globular protein can fold reliably into a particular three-dimensional conformation in vitro is an important one in biophysics. If we wish not only to predict the folded state of a protein, but also to understand the folding phenomenon in terms of physical processes, we need to use physics-based methods: we run computer simulations that attempt to mimic the behaviour of protein molecules. A complete quantum mechanical simulation is not yet possible. But when we attempt to design simplified models, we face the problem of how to simplify: which characteristics are essential and which can be ignored? What effective energy functions does this imply for the simplified system? Folding experiments on small globular proteins show evidence of thermodynamic and kinetic co-operativity, which can be thought of as indicating a phase transition between native and denatured states. We can use this observation to constrain the set of simplified models and interaction schemes: for a particular model to be a valid simplification, it must produce co-operative behaviour when applied to a suitable small globular protein. In this work we ask which simplified models lead to thermodynamic co-operativity. We apply Langevin dynamics to a coarse-grained off-lattice model, representing the protein as a string of residues in a continuum space. The effective energy function is native-centric. It can include both non-local interactions (pairwise attractions between residues far apart along the chain) and local interactions (interactions between near neighbours, represented by effective dihedral and angular forces). Our results show that both local and non-local interactions are important for thermodynamic co-operativity. We discuss the implications of this conclusion for computational models and for our understanding of protein folding.

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## 2-25

### Limits in the sensitivity of metabolic cascades

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Covalent modification cycles and cascades thereof are ubiquitous in metabolism and signal transduction. Theoretical studies have suggested that they may serve to increase the sensitivity of the system. However this has not been corroborated experimentally in vivo. Here we demonstrate that the assumptions of the theoretical studies, i.e. irreversibility, and absence of product inhibition, were not trivial: when the conversion reactions are close to equilibrium or saturated by their product, the zero-order ultrasensitivity disappears. For high sensitivities to arise, it is necessary to have not only substrate saturation (zero-order) but also to have high equilibrium constants and low product saturation. Many covalent modification cycles are catalyzed by one bifunctional ambiguous enzyme rather than by two independent proteins. We show that this makes the conditions of high substrate concentration and low product concentration for both reactions of the cycle internally inconsistent: such modification cycles cannot have high responses. With this finding we predict that in glutamine synthethase (GS) cascade if the ratio PII/PII-UMP is maintained constant, an insensitive response for concentrations in the GS cascade will be obtained, what explains the unexpected experimental evidence reported. (Ortega F.,Acerenza L., Westerhoff H., Mas F. and Cascante M, Proc Natl Acad Sci U S A 2002 Feb 5;99(3):1170-5). This finding rationalizes the absence of strong phenotypes for many signal-transduction proteins.

## 2-26

### Analysis of the relationships between the parameters of the electric pulse necessary for cell electroporation

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Theoretical relationships between the parameters of the electric pulse necessary for the poration of the cell have been obtained. Analysis has been made assuming that pore formation and disappearance are random processes [1].
First, the relationships between the external electric field strength required to porate cell, E_p, and the length of the square wave and exponential pulses, tau_i, were studied. It has been obtained that the cell poration time depends on the pulse intensity: the shorter the pulse length, the higher the field strength should be. This dependence is much more pronounced for short pulses (tau_i<10 tau_m). For long pulses (tau_i>10 tau_m) the electric field strength required for the poration of cell membranes increases significantly slower with decreasing pulse.
Second, taking into account that the generated transmembrane potential depends on the frequency of an electric field, the relationships between E_p and the frequency of the applied ac field, *f*, were calculated. It has been obtained that although the electric field strength for electroporation, E_p, is constant for frequencies less than 10 kHz but its value depends on the pulse duration and decreases with increasing tau_i. At higher frequencies (*f* >1/2 pi tau_m) E_p is dependent on the frequency of the ac field.
Dependences obtained theoretically are in accordance with experimental observations [2,3].

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## 2-27

### Retinal isomerization in terms of quantum chemistry and molecular dynamics

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The chromophore retinal of proteins bacteriorhodopsin and visual rhodopsin was studied. Various electronic states were investigated. The izomerisation barriers were found for ground and excited states using by SCF method. Then molecular dynamics method was applied. The isomerization of retinal in model protein environment has been obtained. The collective degrees of freedom were found to take place during retinal isomerization. This degree of freedom allow retinal to turn from cis to trans form and back. It was shown that the process of visual perception can be considered as a cycle similar to the Carno cycle for the heat engine.

## 2-28

### Binocular stochastic models for suprathreshold luminance changes

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We tested the dynamics of neural-conduction time at the large scale of visual perception for the human binocular system. Circular random step-wise achromatic pulses were presented on a colour monitor at a 2-deg field size. Their luminance was randomly selected between 3 and 27 candela per square meter (cd/m2) in increments of 2 cd/m2. A 15 cd/m2 achromatic reference stimuli was selected to provide suprathreshold luminance variations. For this arrangement, manual simple visual reaction times (VRT) were measured at the fovea under monocular and binocular observational conditions. Three human observers took part in the experiment. To examine the rate at which responses were produced in each instant following stimulus presentation (events per millisecond), conditional probability density functions (commonly called hazard functions) were calculated from VRT raw data for each observational condition. Comparing both kinds of functions, the results for all observers confirm that time-homogeneous models, time-dependent Poisson models and models based on a time-nonhomogeneous diffusion process, cannot take into account binocular hazard functions under a wide variety of binocular combination rules supported on a Minkowski-type metric. These results suggest that binocular vision could be viewed as space distributed more than as a point-process, showing the necessity of developing new methods to incorporate the dynamics of the retino-geniculo-cortical pathways.

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## 2-29

**The microcanonical approach to the theory of helix-coil transition in heterogeneous biopolymers.**

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As in previous papers on homopolymer DNA melting [1, 2] theory of thermal denaturation of heteropolymer DNA based on Potts-like model with many-particle interactions is offered. In the framework of transfer-matrix approach we use microcanonical method [3]. The main idea of this method is in substitution of the quenched free energy by annealed one, averaged over the ensemble with constant GC content. On the basis of the homopolymeric characteristic equation the same was constructed for heteropolymer. In first order approximation the melting temperature as linear combination of the homopolymeric ones is obtained. In second order approximation the melting interval as a quadratic function of the T(GC)-T(AT) is obtained. These results coincide with well-known results [4] and offered approach allows to obtain more precise expressions if take into account third, etc. orders. Moreover, for block DNA the quadratic dependence for interval changed to linear. So it has been shown that the earlier offered homopolymeric model is consistent to describe the helix-coil transition in heteropolymers as well.

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## 2-30

**Using the fractal description to explain some properties of proteins**

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The concept of fractal can be applied for a number of properties of proteins. The fractal description of the properties of proteins in not always clear but it could offer a striking explanation of their irregular behavior and for the anomalies in their structures.
This paper presents a complex study made for a great number of proteins. Its aim is to reveal the fact that the proteins present fractal aspects of their structures and dynamics and to compare the fractal dimensions for different elements of the secondary structure.
In order to reveal the fractal aspects of the proteins structures and dynamics the following concepts are used: the fractal dimension of the protein backbone, the scaling exponent obtained using the detrended fluctuations analysis (DFA) method and the spectral coefficient obtained using the spectral analysis (SA) method. These quantities are determined and compared for the free proteins and for some of their complexes with ligands and also for different elements of the secondary structure. The results are interpreted in terms of protein folding and unfolding and the relationship between the structure of the protein and its biological function.

## 2-31

**Theoretical study of comb-polymers adsorption on solid surfaces: its role in capillary electrophoresis**

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We present a theoretical Mean-Field (MF) model of comb-like polymers adsorption from dilute or semi-dilute solutions onto a solid surface, applied to the different cases where the backbone and the side-chains of the co-polymer are either preferably adsorbed or repelled by the surface.
Our study is particularly relevant in the framework of capillary electrophoresis, one of the major separating methods of biological macromolecules. Separation performances are increased by coating the inner surface of the capillaries with neutral polymers. This method allows to screen the surface charges, which are responsible for the adhesion of charged macromolecules (e.g. proteins) to the capillary walls and for the occurrence of electro-osmosis phenomena.
For instance, the use of comb-like neutral polymers - with an hydrophilic backbone and hydrophobic anchored side-chains --as sieving matrices and coating agents, allows to achieve high resolution, but the mechanism regulating both the co-polymer adsorption and the intrinsic structure of the adsorbed layer is still theoretically poorly understood.
We investigate this phenomena and derive explicit asymptotic forms for the monomer concentration profiles, for the adsorbing energy per monomer and for the cross-over distance between the loops-dominated region at the surface and the outer region, dominated by tails and by non-adsorbed side-chains. Thanks to scaling arguments, we determine the threshold between mushroom and brush configurations for the adsorbed combs as a function of the backbone and side-chains molecular weights and of the grafting density per chain.

## 2-32

**Numerical simulations of the raman spectra of gc watson-crick and protonated hoogsteen base pairs**

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Large changes in the Raman spectra of calf-thymus DNA were observed upon lowering the pH. In order to get a better insight into these effects several simulations of the Raman spectra of the guanine-cytosine Watson-Crick and Hoogsteen base pairs have been performed. The Raman spectra of the guanine-cytosine base pairs were computed using the GAMESS package. The first simulation was performed at Hartree–Fock level, using the MINI basis set. Guanine, cytosine and the sugar pucker were included in the calculation. The second simulation of the Raman spectra for the GC base pairs was performed using the DFT calculation. Becke's exchange-correlation functional (B3LYP) and the basis set 6-31G** were employed. In this case, the sugar pucker was not included in the calculation .
By comparing the Raman bands of the GC base pairs in calf-thymus DNA at high and low pH with the theoretical simulations of GC base pairs, it was found that the intensity changes in the theoretical bands located between 400–1000 $cm^{-1}$ are small compared to the experimental ones. The behavior of the cytosine band at 1257 $cm^{-1}$ upon lowering the pH was not reproduced in the GC theoretical spectra. The bands located above 1300 $cm^{-1}$ in the theoretical spectra display intensity changes which are similar with those found for GC base pairs in calf-thymus DNA spectra.

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## 2-33

### Quantum theory of brain organization of mental activity

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Combination of scientific disciplines such as Physics, Chemistry, Biology and Psychology would be able to reveal operational mechanism of brain organization of mental activity in healthy subjects and mental patients by using a new scientific language of contemporary natural science. With special reference to methodology, accumulation of knowledge after many years of theoretical research, clinical examinations of mental patients and healthy persons made it possible to bring forward a new concept – "quantum theory of brain organization of mental activity". The brain organization of mental activity is a quantum field formation and corresponds to equation "molecule = cell = brain =aperiodic crystal". The laws of quantum mechanics act at molecular and atomic levels of mortal material. Therefore, after corresponding transformations and innovations (dissymmetry, irreversibility, energy - informative non-ordinary of vital system) in formal apparatus of quantum physics we have carried out theoretical analysis of brain activity at micro and macro levels. At molecular level the developed quantum-dynamic models of DNA and protein confirmed the fact that the intracellular system represents proton-electron system of conductivity. By proton-electron conductivity we have been carrying out all biochemical processes of conformation and configuration. This quantum system is characterized by such notions as energy conditions and transitions, potential barrier and tunneling, quantum jump and expectation time. In conclusion, it is noteworthy to mention that the new approach "quantum understanding" to mechanism of brain function and structure, and mental activity may represent highly promising trends in the development of biophysics, neuroscience and biomedicine.

## 2-34

### Dynamical motions of the mechanosensitive channel MscL

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The mechanosensitive channel (MscL) is an integral membrane protein which opens in response to membrane tension. Physiological data have shown that the gating transition involves a very large conformational change, and that the open state of the channel forms a large non-specific pore with a high conductance. Starting with the X-ray structure of the MscL from *Mycobacterium tuberculosis*, the *Escherichia coli* MscL structure was first modelled by homology modelling. Then, the dynamical and conformational properties of the channel were explored, using molecular dynamics and normal mode analysis. The normal mode calculations were led in order to identify principal motions of the structure and putative key regions implied in the gating mechanism. Such an analysis was also performed on putative opened intermediate structures. Similar dynamical behaviors (twist and tilt motions) are observed, characteristic of the channel architecture, but subtle differences exist being due to the different relative positioning of the structural elements. Molecular dynamics simulations were led on the MscL embedded in a fully hydrated phospholipid bilayer. The exploration of collective motions was.perfomed using the essential dynamics method. A comparison of the results obtained with the two kinds of method (normal mode analysis and essential dynamics) may highlight the dynamical properties associated with the intrinsic channel structure, and the ones resulting of the influence of the surrounded bilayer. The ability of particular regions of the channel to deform will be discussed with respect to the functional and structural properties put into evidence by numerous experimental data.

## 2-35

### Molecular dynamics studies of lipid bilayers: major artifacts due to truncating electrostatic interactions

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The aim of this work [1] is to examine the influence of truncating the electrostatic interactions in a fully hydrated pure dipalmitoylphosphatidylcholine (DPPC) bilayer through 40 ns molecular dynamics simulations. Simulations in which the electrostatic interactions were truncated are compared to similar studies using the Particle-Mesh Ewald (PME) technique. We find that all examined truncation distances (1.8 to 2.5 nm) lead to major effects on both structural and dynamic properties of lipid bilayers, such as enhanced order of acyl chains and reduced lateral diffusion coefficients. The results obtained using PME, on the other hand, are consistent with experiments. These artifacts are interpreted in terms of radial distribution functions (RDFs) of molecules and molecular groups in the plane of the bilayer. Pronounced maxima or minima in the RDF appear exactly at the cutoff distance indicating that the truncation gives rise to artificial ordering between the polar phosphatidyl and choline groups of the DPPC molecules. In systems described using PME, such artificial ordering is not present. We can conclude that the truncation of electrostatic interactions gives rise to artificial order in the plane of the membrane, thus changing the phase behavior of the lipid bilayer.

## 2-36

### The side-chain conformation distribution of enkephalin affects its selectivity for mu and delta opiate receptors

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A long-standing problem in neuropeptide research has been to determine the bioactive conformations of enkephalin. In the present work, a link is proposed between the probability distribution of Leu- and Met-enkephalin's side-chain conformations and the selectivity of these endogenous neuropeptides for the mu and delta opiate receptors. To find the probability distribution of enkephalin's side-chain conformations, clustering methods were applied to analyze the molecular dynamics trajectories of Leu- and Met-enkephalin in water. After a short transient, the backbone of both enkephalin species reached equilibrium in the single bend conformation, in agreement with some recent experiments (Salim et al. 2003, J Phys: Condens Matter, in press). The side-chains, however, exhibited high mobility throughout the simulation, so to determine whether side-chain equilibrium distributions could be identified, clustering analysis of the Tyr-to-Phe ring center distance was used while the enkephalin backbone was locked in the single bend state. It was found that Leu-enkephalin has at least 3 distinguishable clusters with varying probability, whereas Met-enkephalin only has 2. The Tyr-to-Phe ring center distance in Leu- covers a wider range than in Met-enkephalin (5-15 and 8-15 Angstrom respectively). This observation is used in combination with the identified clusters to predict that the selectivity of Leu for the mu-receptor will be lower than Met-enkephalin’s selectivity for this receptor (by a factor 0.7), and also that the selectivity of Leu and Met for the delta-receptor are approximately equal. These predictions are in good agreement with experiment (Kosterlitz 1985, Phil Trans R Soc Lond B, 308, 291-297).

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## 2-37

**A kinetic theory of active transport based on the stochastic energization-relaxation channel model**

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The stochastic energization-relaxation channel model developed by Muneyuki et. al [1] explains the vectoriality of active transport via random switches of an ion pump between two states differing in the free energy profiles that govern the reaction steps of ion translocation. These stochastic jumps hinge on external energy supply and, as shown by computer simulations [1], they induce unidirectional ion transport with properties in qualitative agreement with experiments. We provide an analytic formulation of this theory, in which the energization-relaxation switches are described by a stationary dichotomous Markov process. The kinetic equations for the average mole fractions of pump states with different ion occupancies are easily obtained from those in the absence of jumps. By numerically solving the associated eigenvalue problem, the solution of the system is obtained as a linear combination of exponentials, and the pump flux is calculated. It vanishes in the absence of switches. We apply the formalism to a model proton pump under different conditions of pH and membrane potential, comparing the simple case of a channel-like pump with three proton binding sites and single ion occupancy with the case of multiple occupancy. Only the latter is able to reproduce the experimentally observed pH-dependency of the pump current. The results obtained at different membrane potentials provide insights into the voltage drops between ion binding sites.

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## 2-38

**A model for protein conformational changes**

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Proteins act as the machines of life, they drive essentially all the physical and chemical processes that go on in living cells: they catalyse reactions, pass signals and provide basic structure. Although they are 100 million times smaller than man-made machines they can perform similar tasks such as transport molecules from one part of a cell to another and act as motors. The way they function is still very obscure but it is known that they perform their functions by going from one conformation into another. The possibility that vibrational energy transfer is a step in protein function was proposed in the early 1970's in connection with a mechanism for muscle contraction [1]. Computer simulations show that vibrational energy transfer is indeed a robust way of transferring energy from the active site of a protein to other regions where the energy is used for work [2]. Recent experiments lead to a lifetime of 15 ps for such a vibration in myoglobin [3], and of 35 ps in the organic crystal of acetanalide [4], two orders of magnitude larger than previously thought. Still missing is a model of a mechanism by which vibrational excited states can lead to protein conformational changes. and protein folding [5].Here results from such a model will be presented.

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## 2-39

**Molecular dynamics simulations of a DPPC bilayer with a gromos parameter set optimized for long alkanes**

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Although force fields are parameterized to reproduce both molecular-orbital calculations in gas phase and experimental data in condensed phases, there is no guarantee they will give good results for every system. An example: the GROMOS 43A2 set (optimized for small alkanes) gave wrong densities when used in simulations of aliphatic alkanes with chains longer than $C_7$. In contrast, the 45A3 set was successful in simulations of these longer alkanes (up to $C_{20}$) [1]. To test its performance in a phospholipid assembly at the liquid crystalline phase we performed a 14 nsec simulation of a hydrated 128 DPPC bilayer. Electrostatic interactions were calculated with the particle-mesh Ewald algorithm. It was necessary to impose a –90 atm lateral pressure to prevent a transition to crystalline phase. During the first 6 nsec of simulation, the area per lipid oscillated around the experimental value, as well as time averaged bilayer thickness, order parameters of the lipid tails and headgroup orientation. In the last 8 nsec, the area decreased progressively with an apparent leveling off at a smaller value (but within experimental error). This new parameter set was able to simulate a stable DPPC bilayer in the liquid crystalline phase, provided a net surface tension was imposed. Also, this study stresses the importance of long simulations of lipid assemblies.

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## 2-40

**How does a protein find its site on the DNA?**

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Recognition and binding of specific sites on the DNA by proteins is central for many cellular functions such as transcription, replication, and recombination. In the process of recognition, a protein rapidly searches for its specific site on a long DNA molecule and then strongly binds this site. Here we aim to find a mechanism that can provides both fast search (~1 sec) and high stability of the protein-DNA complex ($Kd=10^{-15}-10^{-8}$ M).
Earlier studies have suggested that rapid search involves sliding of a protein along the DNA. We consider sliding as a one-dimensional diffusion in rough (random) energy landscape formed by the energy of binding to consecutive sites on the DNA. Our analytical results and computer simulations demonstrate that, in spite of the landscape roughness, rapid search can be achieved by combination of 1D and 3D diffusion. We estimate a narrow range of the specific and non-specific DNA-binding energy required for rapid search. However, realistic energy functions can not provide both rapid search and strong binding. To reconcile these two fundamental requirements we introduce a novel search-binding mechanism that involves coupling of protein binding and protein folding.
Our mechanism is supported by several experimental evidences and known structures of protein-DNA complexes. Our model of protein-DNA recognition suggests new experiments and novel approaches for bioinformatic prediction of DNA-binding sites.

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## 2-41

### Influence of treadmilling on the dynamics of active filament bundles

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The cytoskeleton is a complex network of protein filaments which determines the mechanical properties of eucaryotic cells. Stressfibers and contractile rings are important substructures consisting of aligned filaments. These essentially one-dimensional assemblies actively generate tension through the interaction between filaments and molecular motors. Further dynamics is generated by polymerization and depolymerization of filaments.

Here we study the dynamics of active bundles of polar filaments subject to polymerization and depolymerization. The dynamics stems from relative displacements between the filaments induced by active crosslinks. In addition, polymerization and depolymerization leads to treadmilling of the filaments. In the presence of passive crosslinkers this results in relative displacements as well.
We develop a generic description for the dynamics of such active bundles, where we assume that two filament interactions dominate.

The dynamic equations are analyzed by investigating the stability of the homgeneous state.It is unstable when the interaction between filaments of the same orientation exceeds a critical value. Treadmilling decreases this critical value so that the homogenous state may be unstable even without any interaction between equally oriented filaments. Asymptotically, the bundle assumes either a homogeneous or inhomogeneous stationary state, or forms a travelling or oscillating wave. Several of these states may coexist.

## 2-42

### Three-dimensional model for fluorescence recovery after photobleaching experiments with confocal laser scanning microscopes

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Fluorescence recovery after photobleaching (FRAP) experiments started 30 years ago to visualize molecular mobility in living cells. Estimation of diffusion coefficients by FRAP relies largely on a model proposed by Axelrod in 1976. However, some of the assumptions of this model are inappropriate when FRAP analysis is performed with a confocal laser scanning microscope resulting in an incorrect estimation of the diffusion coefficients.
In confocal FRAP, assumption of a two-dimensional recovery is not valid as the effective radius of the bleaching beam is only slightly wider in the axial direction than in the radial direction. Also, for species with high mobility, diffusion during scan cannot be neglected. Furthermore, there is always a significant time required to switch the laser intensity between the bleaching and scanning modes.
We developed a new model which overcomes these problems. We tested this model by solving numerically the three dimensional diffusion equation with a bleach term added, generating simulated FRAP recovery curves for a wide range of diffusion coefficients and immobile fractions. Experiments are underway to determine the mobility of purified dextrans in aqueous solution and these results will be discussed, checking their agreement with expected values according to the Stokes-Einstein equation.

## *Posters*
*- Emerging techniques -*

## 3-1

### Detergent concentration measurement by droplet shape analysis

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Fast and accurate detergent concentration measurement is a prerequisite to understand the membrane protein reconstitution into proteoliposomes. While elegant lipid and protein assays are widely used, techniques to determine detergent concentrations are laborious. Radioactive labeled detergents yield exact values but are expensive and not commercially available. The falling droplet technique can be used with any detergent but it is slow and consumes significant sample volumes. When using the sitting droplet method, only small sample volumes are required, and results are quickly available. Commercial drop analysis instruments can be purchased but did not fulfill our requirements. To characterize the droplet shape we have developed a fast and cost effective solution using video cameras looking at two different angles and a software fitting a parabola to the droplet. The distance between the top of the droplet and the focus of the parabola is a well-behaved function of the surface tension. To eliminate reflections/refraction effects inherent to droplets having a high surface tension (e.g. water) and to enhance the contrast prior to image acquisition and image processing, the illumination was optimized. Replaceable paraffin film is used to achieve reproducible liquid-solid interaction. Operation is fast and easy to perform, yielding an accuracy similar to that achieved with radioactively labeled detergents. Many detergents (e.g. OG, OTG, DDM, DM, Octyl-POE, UDM, UTM, SDS, SLS, TX100) and the corresponding binary detergent-lipid or detergent-protein mixtures were characterized using this technique.

## 3-2

### Multiparametric color-changing fluorescence probes and their application in molecular and cellular research

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A series of novel fluorescence probes was developed with unique ability to provide multiparametric description of the properties of their binding sites in proteins and biomembranes. They are based on 3-hydroxychromone and 3-hydroxyflavone dyes that are able to exist in several spectroscopically distinguished ground-state and excited-state forms. These dye molecules can serve simultaneously as polarity, electronic polarizability, electric fields and hydrogen bonding sensors due to differential response of their different spectroscopic parameters. The most significant component of this response is the change of emission color, from blue-green to orange-red that occurs due to the existence of Excited-State Intramolecular Proton Transfer reaction. The proper chemical modifications of these dyes allows achieving the site-specific and covalent binding to proteins, chelation of ions and vectorial insertion into biological membranes with the strong response to polarity, hydration and dipole potential.

## 3-3

### Fluorescence imaging as a screening technique of photosynthetic membrane activity

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The high resolution multi-colour fluorescence imaging system (FIS) ia a new technique which offers the possibility to study the distribution and irregularities of fluorescence signatures over the whole leaf area by simultaneously screen several thousand points of intact leaves. The Karlsruhe FL-FIS instrument is developed by replacing the expensive laser by a pulsed flash light (FL). The wavelength bands for fluorescence excitation can be selected by appropriate filters. Chlorophyll fluorescence images at maximum of fluorescence Fp and steady-state at fluorescence Fs, as well as the subsequent determination of the image of fluorescence decrease ratio, RFd-values, allow to screen gradients and small local damages of photosynthetic activity. Integrating the blue and green fluorescence of cell walls as internal fluorescence standards, permit easily and very early detection of strain and stress to the photosynthetic apparatus. Fluorescence at the four bands and Chl fluorescence yield of sun and shade leaves of some tree species were determined. The considerable differences and gradients of the Chl fluorescence over the leaf area at Fp and Fs as well as RFd-images demonstrated that the activity of photosynthetic membranes of shade leaves is distinctively lower than those of the sun leaves. The fluorescence ratios blue/red and blue/far-red prove to be very sensitive to variations of the illumination on growth. The results with the fluorescence imaging of leaves demonstrate that the new FL-FIS is a superior technique which allow to screen the patchiness and gradients over the leaf area and to detect local disturbances in activity of photosynthetic membranes.

## 3-4

### Electronic detection of DNA by field effect on transistor arrays

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The research for an efficient interface between molecular biology and microelectronics represents a very active and challenging field, both in terms of applications and basic science. Among the various approaches, an appealing concept is the in-vitro detection of specific interactions between biomolecules on the surface of field effect transistors (FET). These hybrid systems are rather complex and in most cases a quantitative understanding of the transduction of a biological event into an electrical signal is still missing. We have developed an approach to electronically detect charge variations at a Si02-electrolyte interface, using linear FET arrays. We have investigated the dc field effect response of transistors to adsorption of Poly(L-lysine) and DNA. Local deposition of these two biopolymers of opposite charge induces reverse variations in the dc current-voltage characteristics of the transistors. The differential signals are studied as a function of electrolyte salt concentration and biopolymer concentration and compared to an analytical description of the hybrid system (semiconductor / biopolymers / electrolyte) which includes screening in the electrolyte in the frame of the Gouy-Chapman theory. We have performed experiments on single stranded DNA (oligonucleotides) and double stranded DNA (PCR products) of different length. Using Cy5-modified DNA the electronic signals are compared with local fluorescence measurements. Our detection scheme is compatible with biological protocols on genomic DNA samples : we have combined the electronic approach with allele specific PCR and demonstrate detection of a single base pair mutation.

## *Posters*
*- Emerging techniques -*

## 3-5

### Cell-Free Expression of Membrane Proteins: Preparative Scale Production and Labeling of Multidrug Transporters

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Functional and structural studies of membrane proteins are often limited by insufficient production rates in conventional bacterial or eucaryotic expression systems. Low yields are usually caused by protein instability, rapid degradation or toxic effects to the host cells. In order to circumvent these problems, we have analyzed the efficiency of membrane protein production in a T7 based cell-free expression approach using an E. coli S30 cell extract in a coupled transcription-translation system. Dialysis of the reaction mixture containing all enzymes against a feeding solution providing low-molecular-weight substrates and precursors extended the protein synthesis for more than 10 hours. First, all reaction components including the S30 extract preparation protocol were optimized and several milligrams of protein could be obtained from a single milliliter of reaction mixture. In order to provide a suitable environment for membrane proteins, the cell-free expression system was optimized for the supplementation of various hydrophobic additives like micelles, liposomes or chaperones to assist protein folding. Several highly hydrophobic small multidrug transporter proteins of the Smr family have been expressed and the productivity of the cell-free system was found to be superior compared with the expression in E. coli cells. We demonstrate the production of up to milligram quantities of membrane proteins per milliliter of reaction mixture, and the isolated proteins were found to be active as analyzed by specific transport assays. Hence, an efficient uniform or amino acid type specific labeling with stable isotopes of membrane proteins is feasible by cell-free expression.

## 3-6

### Micromanipulation of celluar biomaterials with dynamic holographic optical tweezers

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The dynamic holographic optical tweezers (DHOT) technique can produce one to hundreds of uniquely arranged optical traps in two-dimensions, as well as more limited arrangements in three-dimensional volumes. The method works by transforming a single laser beam into multiple propagating beams with a phase-only diffractive optical element (DOE) designed to yield the desired optical landscape. With a real-time reconfigurable DOE such as a spatial light modulator (SLM), the optical traps are mobilized and independently steered to arrange biological materials and to exert coordinated calibrated forces. This new method will be introduced with a focus on biophysical applications.

## 3-7

### Conformations of separate biological molecule in condense phase probed by seira

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It is known during last 20 years that molecules adsorbed on island metal film or colloidal metal particles show 10-1000 times more intensive IR absorption than those in conventional geometry without metals [1]. This effect was named SEIRA - surface enhanced infrared absorption. Earlier we have applied SEIRA for study of nucleic acid damage induced by cancer process and proved high sensitivity of this method [2]. We studied 2 colloidal aqueous solution of C60 [3] and obtained that due SEIRA we have registered spectra of separate molecules of C60 or their clusters. Now we apply SEIRA to study of conformational state of different proteins and phopholipids whose sizes are similar to metal roughness (close to 50-200A). Conformation states of the molecules could be recorded after its precipitation on metal substrate as well after drying that give a possibility to compare their states after drying. We used Amid I and Amid II for determination of the conformation states of the protein. It was shown that it is better to use rough metal surface than colloidal particles for conformational study of proteins. New conformation states of the protein could be induced by colloidal particles. Influence of thickness of protein film on enhancement of SEIRA and validity of Bugger-Beer low are discussed also. 1. V.A. Kosobukin, SURFASE. Physics, chemistry, mecanics, 1983, 12, 5-20. 2. G.I. Dovbeshko, V.I. Chegel, N.Ya. Gridina et. al., Biopolymers (Biospectroscopy), 2002, 67(6), 470-487. 3.G.V. Andrievsky, V.K. Klochkov, A.B. Bordyuh, G.I. Dovbeshko. Chem. Phys. Let. 2002, 364(1-2), 8-17.

## 3-8

### Numerical simulation of speckled light scattered by red blood cells in suspensions

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The present contribution focuses on numerical simulation of time dependent speckle pattern obtained by stochastic interference of light coherently scattered by human red blood cells. The speckled light scattered by biological cells provide information on both static and dynamic parameters characterizing the investigated sample. Software able to simulate light scattering experiments having as input data the main parameters of the scattering centers and the geometry of the experimental configuration was developed. For different scatterers density the software generates the interference image with a controlled spatial resolution and also the interference pattern dynamics. Two processes give the red blood cells dynamics: the sedimentation in gravitational field, simulated by a Stokes dynamics and an additional random Brownian motion. The random dynamics was introduced by uncorrelated random Brownian velocities distributed isotropically and by randomly distributed angular velocities, giving the dynamics of the azimuthal angle.
From simulated images one obtain the whole speckle pattern and also its detailed characterization. Local interference maxima are identified and for each speckle (local maximum) the program give the maximum light intensity and the speckle area. The stochastic properties of the results originate from the random distribution of scattering centers.
The simulated data given by the software can be compared with experimental data, the similarity being a potential tool for identification of relevant parameters.

## *Posters*
*- Emerging techniques -*

## 3-9

**Yttrium hydride films as hydrogen sensors**

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Yttrium and rare-earth hydrides show a drastic change in their optical properties upon hydrogen concentration. $YH_2$ is a metal with no transmittance in the visible range whereas $YH_3$ is an insulator with a 75%-90% transmittance. Such a dramatic optical transition makes these materials good candidates to develop optoelectronic devices, as hydrogen sensors, optical modulators or switchable mirrors.
In this work we study the application of yttrium hydride thin films as hydrogen sensors. The yttrium films are prepared under ultra-high vacuum conditions and covered with a palladium (Pd) layer that prevents yttrium oxidation, while being permeable to hydrogen. Two different hydrogenation processes are considered: i) pressured hydrogen and ii) electrochemical hydrogenation. We analyze the effect of the yttrium layer thickness, and the hydrogenating conditions on the hydrogen sensitivity of the films.
We find that yttrium layers of hundreds nanometers are more suitable to develop hydrogen sensors than thinner samples, despite their lower optical transmittance. Electrochemical hydrogenation enhances significantly the optical switching of these films. We have developed an electrochemical cell consisting on an electrolytic solution of 0.01 M KOH placed between a 150 nm yttrium/15 nm Pd cathode, and a 15 nm Pd anode. A small voltage (1.5 V) is enough to quickly hydrogenate the yttrium film. Dehydrogenation, characterized by the loss of film transparency, starts as soon as we invert the polarity. Other electrolytes can also be used. This device could be applied to detect the presence of ions of biological importance, such as $K^+$.

TYPE OF COMMUNICATION: Poster

## 3-10

**Towards engineering organs by cell aggregate printing**

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Aggregates of living cells (i.e. model tissue fragments) under appropriate conditions fuse like liquid drops. According to Steinberg's differential adhesion hypothesis (DAH), this may be understood by assuming that cells (i) are motile and (ii) possess cell specific adhesion apparatus. Here we report results of experimental and modeling efforts to construct three-dimensional cellular structures of prescribed shape by a novel method: cell aggregate printing. Spherical aggregates of similar size made of cells with known adhesive properties were prepared. Aggregates were embedded into biocompatible gels. When the cellular and gel properties, as well as the symmetry of the initial configuration were appropriately adjusted the contiguous aggregates fused into ring- and tube-like organ structures. To elucidate the driving force and optimal conditions for this pattern formation, Monte Carlo simulations based on a DAH motivated model were performed. The simulations reproduced the experimentally observed cellular arrangements and revealed that the control parameter of pattern evolution is the gel-tissue interfacial tension, an experimentally accessible parameter.

## 3-11

**A new infrared window (5-200 microns) for the study of metal - ligands interactions at the redox active sites of metalloproteins**

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Infrared difference spectroscopy is a powerful tool to analyze at the molecular level the properties of amino acid side chains or cofactors at the active sites of proteins. Until now, most of the information obtained by this technique concerned the mid-infrared domain (5 – 10 microns). However, to determine the structural factors governing metal sites specificity and reactivity in metalloenzymes, it is worth to extend the analysis to the far infrared region, where metal-ligands IR modes contribute.
In this spectral domain, almost all information available is obtained by resonance Raman spectroscopy. Only metal sites and oxidation states bearing an absorption spectrum in the visible range are accessible to this technique. Moreover, vibrational modes will dominate the spectra, which are enhanced by the charge transfer interaction. Infrared spectroscopy, probes the vibrational modes of metal centers independently of their optical properties.
To extend the vibrational information to metal sites or modes not enhanced by resonance Raman, we developed a set-up to access the far infrared region, using electrochemistry – coupled to Fourier transform infrared spectroscopy. The power of this approach, its validity and the specific information obtained on metal-ligands interactions will be illustrated with the Fe-SCys4 center of rubredoxin, Cu-NHis4 center of Cu,Zn-superoxyde dismutase, and cytochrome c. Using 15N and 65Cu labeling, we identified the Cu-His stretching modes in Cu,Zn-SOD. We also observe the IR mode of the water molecule present at the active site. Effect of pH and anion binding at the SOD Cu center will be discussed.

## 3-12

**Polymeric multilayers for strain sensing**

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Stacks of dielectric films with low absorption coefficient have been widely used to control and manipulate the spectral behavior of light beams. Such multilayers are present in beam splitters, interference filters or antireflecting coatings. These devices are generally aimed to work under room conditions, hence they are based in inorganic materials such as oxides, etc, which exhibit good thermal behavior and stiffness.
In this work we have fabricated interference filters based in alternating organic polymers, poly(9-vinylcarbazole) and poly(vinyl alcohol), with spectral response centered at a $\lambda_B$ within the visible range. These structures have been developed taken advantage of a fast and cheap deposition technology (spin coating), which yields reproducible thick films of good quality (surface roughness below few nm). The fabricated multilayers exhibit a $(2p+1)\lambda_B/4$ structure (p=1,2), which guarantees a spectral bandwidth around 17 nm , narrower than that obtained by the widely used $\lambda_B/4$ stacks.
Due to their elastic properties, the polymers used become less sensitive to fracture than the inorganic materials. The mentioned optical filters have been fabricated on rigid and flexible substrates, revealing a high sensitivity of their spectral response upon substrate deformations. This behavior is proposed as a principle for strain sensing via the spectral analysis of the transmittance or reflectance of these coatings.

***Posters***

*- Emerging techniques -*

## 3-13

### Laser light scattering on blood – approximate expressions for successive order scattered light fluxes

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The light-scattering techniques are often used to investigate the static or dynamic properties of biologically relevant objects such as cells in suspensions or organized in tissues. The simplest biological systems used to test the theoretical models are the red blood cells. If the light-scattering properties of individual erythrocytes are relatively well understood the spatial and angular distribution of light intensity scattered by red blood cells in suspension is extremely complex and only partly understood.

The main goal of this work is to find approximate simple analytic solutions for the radiative transport equation in any scattering order.

The radiative transport equation can be written as an infinite hierarchy of integro-differential equations for each of the successive order scattered light fluxes. Particles with characteristic dimensions higher than the light wavelength and relative refraction indexes close to one, have a scattering phase function strongly peaked in the forward direction. This peculiar angular dependence was used to approximate the angular dependence of the successive order scattered light fluxes and to obtain simple analytic expressions for any scattering order. A realistic phase function, often used to describe scattering from red blood cells is given by Henyey-Greenstein formula. The natural expansion of the formula in terms of Legendre polynomials was the main ingredient which allowed us to describe successive order scattering fluxes in terms of Henyey-Greenstein phase functions, with a $n$-th order mean cosine given simply by the $n$-th power of the mean cosine of scattering angle.

## *Posters*

*- Charge transfer and bioenergetics -*

## 4-1

### Towards a biomimetic model for hydrogen photoproduction

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The development of a biomimetic model for hydrogen photoproduction is based on the construction of an electron transfer chain. This chain involves photosynthetic membrane proteins (Photosystem I) immobilized on an electrode and soluble proteins like cytochrome c6, ferredoxin and hydrogenase. Light absorbed by Photosystem I delivers the energy necessary for the reaction. This biocatalyst offers several interesting possibilities : control of electron transfer in space by orientated grafting of Photosystem I on the electrode surface, combination of proteins from different origins and measuring of catalytic efficiency. Immobilization of Photosystem I from the cyanobacterium Synechocystis 6803 requires modification by directed mutagenesis to graft it in a stable way and with a well-defined orientation. Functionalization of the electrode surface has also to be optimized for that purpose. First results concern electrochemical characterization in solution of electron transfer between Photosystem I and its natural or artificial redox partners. Electron transfer is studied by enzymatic electrocatalysis, a method based on the coupling between electrochemical and enzymatic reactions and directly observable by cyclic voltammetry. A catalytic photocurrent has been detected upon illumination of such a reaction medium, supporting the functionality of the electron transfer chain. This approach allowed the determination of the kinetic parameters of the electron exchanges between Photosystem I and its soluble partners.

## 4-2

### Organization of mitochondrial coenzymes Q in biomimetic membranes

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Coenzymes Q, or ubiquinones (UQn), are major compounds of the mitochondrial respiratory chain. Apart from this bioenergetics function, ubiquinones act in all cellular membranes as antioxidants. We have studied the behaviour of ubiquinones with different isoprene chain length in phospholipidic model membranes: Langmuir monolayers and multilamellar vesicles, composed of phosphatidyl-choline (PC) or cardiolipin (CL). Analysis of 2D thermodynamic parameters have shown that phospholipids-UQ repulsions increase with the isoprene chain length, and that PC-UQ films are always partially miscible, while CL-UQ mixtures lead to a lateral phase-separation. Observation of monolayers by fluorescence microscopy permitted to note that ubiquinones are soluble in the less-condensed lipidic phase. Grazing incidence X-rays diffraction results showed that the presence of ubiquinone do not perturb the organization of phospholipid molecules and confirmed that strong phospholipids-UQ repulsions lead to the expulsion of ubiquinones from the monolayer, or transversal "phase-separation". Differential scanning calorimetry and Raman spectroscopy allowed to study the influence of small proportions of ubiquinones on the thermotropic phase behavior of PC vesicles. The interpretation of these results allows to propose that short-chain ubiquinones are localized near PC polar heads, laterally organised, while long-chain ones would be rather transversally organised in the center of the bilayer. Thus, in model membranes, ubiquinones localization depends on their isoprene chain length. If a such difference of organization exist in biological membranes, it could be correlated with a different biological action for long-chain and short-chain ubiquinones, concerning their bioenergetics functions or the regulation of their antioxydant properties.

## 4-3

### Localization of coenzyme Q10 and squualane in lipid bilayers by neutron diffraction

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A recently proposed model for proton leakage across cellular membranes (Haines,T.H.,Prog. Lipid Res. 40, 299 [2001]), the "water cluster" model, posits that proton leakage may be inhibited by hydrocarbons packed in the bilayer center. In living cells such leakage consumes ATP energy. We have used neutron diffraction to show that squalane and CoQ10 are resident in the bilayer center. Squalene is a polyisoprene that appears to be used by alkaliphiles to inhibit proton leakage (Clejan et al., J. Bacteriol.,168, 334 [1986]). CoQ10 is a polyisoprene that has a major role as a mitochondrial redox cofactor in oxidative phosphorylation. It may have another role: inhibiting proton leakage across the inner membrane. Although many indirect methods have suggested that CoQ10 lies in the bilayer center, the combined use of deuterium labels and neutron diffraction allows an unambiguous localization at the center. The measurements were made on stacked bilayers of synthetic PC tempered with PS or PG to stabilize the bilayers. Taken together these data suggest that polyisoprenes reside in the bilayer center of straight-chain lipid bilayers. This is consistent with their structure as hinged planes in which each carbon is in one of the planes. Our data suggest that other polyisoprenes such as dolichol in lysosomes and plastoquinone in chloroplasts likewise lie in the bilayer center and may also inhibit proton leakage.

## 4-4

### Charge translocation buring cosubstrate binding and /or transport in the Na+/proline transporter of E.Coli

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Na+/Proline transporter of E. coli (PutP) is a member of Na+/Solute Symporter Family (SSF). PutP is an integral protein of the cytoplasmic membrane and catalyzes the coupled translocation of proline and Na+ with a stoichiometry of 1:1. The coupling ion can also be Li+. In our study, electrogenic property associated with the activity of PutP was investigated by using proteoliposomes containing purified PutP adsorbed onto solid supported membrane (SSM) and applying concentration jumps of Na+ ion and/or proline. The results indicated: 1) PutP is responsible for Na+/proline cotransport, and it is highly specific for proline. Other amino acids, e.g. alanine and glycine can not be transported; 2) The charge translocation which induces transient current response recorded on SSM originates from electrogenic co-substrate binding and conformational change; 3) The Na+ and proline binding sites communicate with each other via conformational alterations. Na+ increases both the apparent affinity of PutP for proline, and the amplitude of proline concentration jump signal; Proline doesn't enhance the apparent affinity of PutP for Na+, but increases the amplitude of Na+ concentration jump signal. In the presence of the cosubstrates, Na+ and proline bind rapidly to the transporter with rate constants > 50 S-1; 4) A remarkable finding is that proline binds to the transporter in the absence of Na+ which was thought to be obligatory for proline binding; 5) Water soluble and membrane-impermeant PCMBS can react with PutP and inhibit its activity.

## *Posters*

*- Charge transfer and bioenergetics -*

## 4-5

**Stoichiometry and energetics of the binding of enlaprilat, captopril and lisinopril to somatic angiotensin converting**

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Angiotensin I converting enzyme (ACE) is a peptidyl dipeptidase that catalyses the conversion of angiotensin I to the vasoconstrictor octapeptide angiotensin II and inactivates the vasodilator nonapeptide bradykinin. The recognition of the role of this enzyme in the regulation of blood pressure stimulated interest in searching for ACE inhibitors for use as antihypertensive agents. In order to determine and quantify the forces driving association of inhibitors and pig lung ACE we have studied the energetics of the binding of enalaprilat, lisinopril and captopril using isothermal titration calorimetry (ITC). The characterization was carried out in different buffer solutions with different heats of ionization in order to determine the number of protons exchanged in the process. The number of protons uptaken can be attributed to an increase in the pK of one or more groups in the complex and the two histidines located in the active site are proposed that may be come protonated in the complex inhibitor-enzyme. Also, this study reveals that the stoichiometry of the binding of these inhibitors to the enzyme is 1:1, and the binding displays a large negative heat capacity change, indicating that non polar forces are primarily responsible of the protein inhibitor interaction. The change in heat capacity change has been explained in terms of change in polar and apolar solvent accessible surface area upon binding. The accessible surface area calculated can be justify for slight conformational changes in the vicinity of the active sites also coupled to binding.

## 4-6

**Transient absortion studies of the Rb. sphaeroides R-26 reaction center in the blue spectral.**

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Transient absorption spectroscopy (with 120 fs time resolution) was applied to measure at room temperature the electron transfer in the reaction center of the purple photosynthetic bacterium Rb. sphaeroides R-26. Absorption changes to an accuracy higher than hitherto achieved were measured in the range from 350 nm up to 720 nm, after excitation with a laser pulse of 80 fs duration at 800 nm. The absorbance changes characteristic of the excited state of the primary donor and extending over the whole spectral range investigated, appeared within 120 fs. Among others, they gave rise to the absorption changes in the blue spectral range, connected with fast electron transfer to bacteriopheophytin. Both the photooxidized primary donor and photoreduced bacteriopheophytin contributed to this effect. Also the slower electron transfer from the reduced bacteriopheophytin to a quinone acceptor was followed due to the transient absorbance changes in the other spectral bands. The wavelength dependence of the fast and slow kinetic in the blue spectral range was investigated and analyzed.

## 4-7

**Identification of metal or cofactor binding sites in soluble proteins monitored using a new microdialysis system coupled with ATR-FTIR difference spec**

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Fourier transform infrared (FTIR) difference spectroscopy is a sound technique to visualise molecular changes at the active site of proteins with a high degree of sensitivity. FTIR difference spectroscopy has notably allowed a better understanding of photosynthetic enzymatic complexes by studying light induced molecular changes. The development of the ATR-FTIR technique introduced new perspectives by replacing the light stimulus by a continuous flow of aqueous buffer over the sample to generate spectral differences. This technique displays several advantages such as the possibility to monitor the pH or buffer composition. Our goal was to extend the application of this powerful technique to the study of proteins in solution. We describe for the first time the coupling of a microdialysis system with the ATR-FTIR technique. With cytochrome c, we demonstrate that our set up enables to record fine structural changes associated to the changes of redox state of the protein as well as the binding of metals or ATP. We observed that Cd2+ or Zn2+ accelerates the oxidation of cytochrome c by H2O2. Further, using the ATR-FTIR set up, we identified a binding site for Zn2+ and Cd2+ involving a histidine side chain and the carboxylate group of at least one glutamate. To provide new insight into the relationship between the binding of ATP to cytochrome c, and the triggering of its release from the mitochondrial membrane during the first steps of apoptosis, we also analyse the binding site of ATP in cytochrome c.

## 4-8

**The flow cytometric measurement of mitochondrial membrane potential in an absolute scale of mV**

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Slow potentiometric dyes together with flow cytometry represent an invaluable tool for monitoring membrane potential of mitochondria. In particular, tetramethylrhodamine methyl ester (TMRM) proved to be suitable for the screening of mitochondrial membrane potential in cultured human skin fibroblast from patients suffering from defects of oxidative phosphorylation [1]. We have developed recently a new method that makes it possible to compare mitochondrial membrane potential and/or its changes in investigated cells and controls in the absolute scale of millivolts. This method combines the flow-cytometric measurements of TMRM fluorescence intensity with a reference signal from MitoTracker Green fluorescence, which provides information on the total number and volume of mitochondria in the investigated cells. Basically, our approach stems from a trivial fact that a logarithmic transformation of Nernst-equation controlled fluorescence intensity of TMRM accumulated in mitochondria leads to a linear scale for mitochondrial membrane potential, which idea was presented in our preceding paper [2]. [1] Floryk D. And Houstek J. (1999) Tetramethyl rhodamine methyl ester (TMRM) is suitable for cytofluorometric measurements of mitochondrial membrane potential in cells treated with digitonin. Biosci. Rep. 19(1), 27-34 [2] Plasek J. et al. (1994) Transmembrane potentials in cells: a DiS-C3(3) assay for relative potentials as an indicator of real changes. Biochim. Biophys. Acta 1196, 181-190.

## *Posters*

*- Charge transfer and dioenergetics -*

## 4-9

**Transient Absorption Spectroscopy of the Excited Flavin Radical in DNA Photolyase from *E. coli***

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Photolyase performs photorepair of photodamaged DNA. Photoactivation is the process of conversion of its FAD cofactor from its singly reduced (neutral radical, FADH°) form in which it exists typically in isolated photolyase, to the fully reduced (catalytically potent) form $FADH^-$. Upon excitation by a visible light quantum, FADH° oxidises a tryptophan (Trp) that is about 14 Å apart[1,2]. A recent study suggests that this electron transfer occurs through a chain of three Trp, and that the first step takes only about 30 ps.[3] We employed femtosecond pump/probe spectroscopy to determine kinetic and spectral properties of photolyase from *E. coli* upon excitation of FADH° by ~200 fs pulses at 620 nm. The spectra showed distinct bleachings at the positions of the ground state absorption bands of FADH°. Excited state absorption was observed below 450 nm and between 650 and 800 nm. The latter band decayed to almost zero with a time constant of about 30 ps which we attribute to the decay of the excited state of FADH°. In the 425 to 600 nm region, after the decay of the 30 ps component, a spectrum persisted with a form that agrees rather well with that found previously for times as late as 10 ns, which can be explained by the formation of $FADH^-$ and a Trp cation radical.[3] The measured spectra allow to estimate the quantum efficiency of the primary electron transfer process in *E. coli* photolyase. For comparison, studies on other systems will be presented, including a mutation of a Trp that is part of the electron transfer chain.

1. Kim, S.-T., Heelis, P. F. & Sancar, A. (1995) *Methods Enzymol.* **258**,

## 4-10

**Electron transfer in the photosyntetic RC**

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Electron Trasfert between the Special Pair and the accessory bacteriochlorophyll in studied in Reaction Center of Rhodobacter Sphaeroides. Calculations are carried out for the wild type and some mutants of the protein. Classical molecular dynamics simulations were used to sample the phase space in order to calculate, via a thermodynamic integration, the free energy differences (or driving force) and activation energies for the process. We compared two different electrostatic models, the first being the simple fixed charge model and the second a model where polarization effects are introduced explicitly via atomic induced dipoles. Although the linearity of the processes, the Gaussian approximation, (derive quantities sampling only one state and using the linear response of the system) can be applied carefully in order to obtain a good agreement for the free energies. Especially in the case of the model with explicit polarization effects, the response of the system can vary from the initial state to the final state giving an erroneous free energy if the Gaussian approximation is used sampling only the initial state. However this model seems superior and more realistic respect to the simple fixed charge model to treat charge transfer processes in inhomogeneous and anisotropic solvents such as proteins.

## 4-11

**Electron Transfer of Cyt-c552 Adsorbed on Mixed Alkanethiols SAM, probe by Surface Enhanced Raman Spectroscopy**

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Cytochrome c552 (Cyt-c552) from Thermus thermophilus bacteria, exhibits structural differences compared to type-c cytochromes (i) the lack of lysine residues close to the heme edge (ii) the presence of hydrophobic beta-sheet surrounding the heme. The surface area of its redox partner (ba3-oxydase) is also uncharged. Hydrophobic interaction seems to be the driving force for the electron transfer between Cyt-c552 and ba3-oxidase. In order to probe the electron transfer of Cyt-c552 in such hydrophobic environment, we are using self-assembled monolayers of alkanethiols coating silver electrode, as a model of surface topography of the ba3-oxydase. Surface Enhanced Resonance Raman Spectroscopy (SERRS) is a powerful method to determine the different conformations of the heme, in both states reduced and oxidized. A quantitative analysis of the SERR spectra, recorded at different potentials, allows to calculate the redox potential of Cyt-c552 adsorbed on the different surfaces and the number of transferred electron (n). On alkanethiols-SAM , there is no efficiency of the electron transfer, n varied from 0.51 to 0.38 depending on the chain length, due to a large denaturation of the secondary structure of the Cyt-c552. Adsorption of Cyt-c552 on mixed alkanethiols and hydroxyl-terminated alkanethiols, gives an homogeneous orientation of the heme (n=1) in its native form and the redox potential is calculated at 0.009 V (ref. SCE). This mixed surface appears to be a good model for the topography of the redox partner. We are now trying to estimate the kinetic constant of the electron transfer using

## 4-12

**Energy transfer by a dithiol-disulfide interchange within membrane-associated enzyme and transport protein complexes in fermenting *Escherichia coli*: accessible SH-groups number of membrane vesicles is increased by ATP and formate**

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The most of bacterial membrane-associated enzymes and transport proteins are known to contain thiol-groups including those with cysteines. These groups displaying in different redox states can determine the affinity and activity of enzyme and transport system [1]. They may also be involved in an energy transfer within the membrane protein complex by a dithiol-disulfide interchange; reducing equivalents are required [2]. In right-side-out and in-side-out membrane vesicles of *E.coli* growing on glucose in the absence or in the presence of external formate (30 mM) under anaerobic conditions at slightly alkaline pH (pH 7.5), the number of accessible SH-groups was considerably (~1.3-1.7-fold for different wild-type strains) increased by ATP (3 mM) but not ADP or stimulated by formate (30 mM), respectively. These effects were inhibited by *N*-ethylmaleimide and absent when *N,N'*-dicyclohexylcarbodiimide or sodium azide was used to inhibit the $F_0F_1$-ATPase as well as in *atp* mutant with deleted $F_0F_1$ or in Cysb21Ala mutant with cysteines in $F_0$ replaced by alanines. Using mutant strains, the increase was shown to be also largely dependent on hydrogenase 4 or hydrogenase 3, main components of formate hydrogenlyase, when bacteria were grown in the absence or in the presence of external formate. The effects were absent for cells grown under aerobic conditions. These results indicate an essential role of SH-groups in the functionality of the $F_0F_1$-

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## 4-13

### Influence of pH and anion binding on the stability of tyrosyl tyrd radical in photosystem II

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Redox active tyrosine residues play important roles in many enzyme mechanisms. The question of how the protein tunes the properties of the highly reactive tyrosyl radicals still needs to be elucidated. The regulation of the pKA of the tyrosine and the charge distribution in the vicinity of the radical are two factors that may be important to understand the formation and the stability of the radical. We have used light-induced FTIR difference and EPR spectroscopies to identify structural factors which determine the stability of the tyrosyl radical TyrD located on the D2 polypeptide of Photosystem II. The neutral radical is formed after light-induced electron transfer from TyrD to the oxidized primary donor of Photosystem and is stable for several hours at physiological pH. However, high pH or the presence of small anions like formate in the environment of TyrD considerably accelerate the reduction of the radical. FTIR difference spectroscopy is particularly useful to probe the protonation state of protein residues. In this work, we analyze the influence of formate and lactate binding on the pKA of TyrD and on the protonation state of residues in interaction with TyrD. Using 13C-labeled formate, we show that formate binds in the vicinity of TyrD, but has no influence on the protonation state of TyrD at physiological pH. FTIR difference spectra obtained at low and high pH in the presence and absence of formate will be compared on samples with labeled and unlabeled tyrosine to determine the pKA of TyrD in both systems.

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*- Nucleic acid and ribosomes -*

## 5-1

**Repression and activation of transcription from an H-NS regulated promoter by modulation of DNA architecture.**

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The chromosomal DNA of E. coli is organized and compacted by the action of nucleoid-associated proteins such as H-NS, HU, IHF and Fis. Next to a role in the organization of the bacterial nucleoid, these proteins have repressive or activatory potential in regulation of transcription. Scanning force microscopy studies indicated that H-NS organizes and compacts DNA by bridging of distant DNA tracts. Although H-NS binds without sequence specificity, it also has the ability to specifically repress transcription by binding to curved regions found in H-NS sensitive promoters. The close proximity of the DNA around the apex of a curve seems to be a determinant for preferential binding of H-NS. If such binding occurs in a promoter region, binding of RNA polymerase is hindered. Repression at the rrnB P1 promoter occurs in a different fashion. H-NS and RNAP bind simultaneously and a transcription competent open complex is formed. However, H-NS holds RNAP trapped at the promoter such that transcription can not take off. SFM experiments indicated that in the open complex DNA is wrapped around RNAP. The DNA arms aside of the bound RNAP are then close, which promotes bridging by H-NS and RNAP thus becomes trapped within a repression loop. Repression of many H-NS sensitive promoters is counteracted by Fis or IHF, which bind directly upstream of the promoter. Novel SFM data indicate that these proteins affect the extent of DNA wrapping around RNAP. We propose that the molecular mechanism underlying relief of repression by these proteins is their effect on the DNA bend angle of the arms aside of the bound RNAP.

## 5-2

**A-form stabilisation of GC-rich DNA in the presence of spermine in Molecular Dynamics Simulations**

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Molecular dynamics simulations of GC-rich DNA in the presence of spermine (4+) have shown that, under sufficient concentrations of spermine, the A-form is stabilised. The concentrations for this conformational effect parallel previous in-vitro experiments. Increases in spermine concentration can reduce the root mean square distance of the DNA fragment from the A-form for time scales exceeding 10 ns. Molecular dynamics trajectories for d(ACCCGCGGGT)2 with various concentrations of spermine were generated using the Amber force field. The spermine : DNA ratio varied from 0:1 to 3:1. We have also examined trajectories of a 34 base pair sequence of poly-G poly-C DNA to establish the effects of spermine binding over a larger scale. All systems were simulated starting from both from canonical A and B-forms. We will discuss the dynamic binding modes of the spermine at different concentrations, as well as its effectiveness in stabilising the A-form and in inducing transitions from B-form to A-form DNA on nanosecond time scales.

## 5-3

**Torsional flexibility as a sensor of oxidative lesion recognition by human OGG1 protein**

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8-oxodG is a powerful mutagenic lesion formed spontaneously in the genome of aerobic organisms and under the action of ionizing radiation. In the course of repair events or due to ionizing radiation exposure, other lesions might occur in close vicinity to 8-oxodG. It has been recently reported that, in this case, repair is strongly inhibited. In this study the relationship between the twisting flexibility of oxidatively damaged 30 bp DNAs and the excision efficiency of the lesion by hOGG1 protein has been investigated. DNA duplexes contained a single oxidative damage or multiple lesions. These latter samples were obtained by annealing a strand containing an 8-oxodG lesion with the complementary one with an abasic site in close opposition to the 8-oxodG. Fluorescence polarization anisotropy (FPA) measurements of ethidium bromide intercalated into the DNA duplexes were performed to evaluate their torsional rigidity constant as well as the hydrodynamic parameters. Binding and cleavage kinetics were allowed using the purified human glycosylase. A dependence of the twisting flexibility of the DNA fragments and of the enzyme efficiency on the relative position of the lesions has been found.

## 5-4

**Oligonucleotide polymorphism single, double, triple and quadruple stranded conformations with the same oligonucleotide**

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We present a study on the polymorphism of the oligonucleotides d(G4A4G4-T4-C4T4C4-T4-G4T4G4) (Oligo1) and d(G4T4G4-T4-G4A4G4-T4-C4T4C4) (Oligo2) using circular dichroism spectroscopy and UV thermal denaturation analysis. These oligonucleotides can adopt several types of conformation: -Single stranded unorganised conformation. -Double stranded conformation with a dangling single stranded arm. -Intramolecular triplex with non isomorphic stretches of G*G:C and T*A:T triplets. For oligonucleotide 1 the third strand (GT) binds parallel to the purine strand of the underlying duplex whereas for oligonucleotide 2 it binds antiparallel. -Quadruplex structure with two dangling duplex arms. The quadruplex part is due to the dimerisation and folding back on itself of the G4T4G4 part of two oligonucleotides. This conformation is observed only with the oligonucleotide 2. Each form exhibits a characteristic circular dichroism spectrum. The transitions between these various conformations have been studied. They depend on the temperature, the nature of the salt (for example Mg++ or K+), the orientation of the third strand, the oligonucleotide concentration. For the oligonucleotide 2 addition of K+ to preformed triplex does not change the structure of the molecule whereas addition and exposure to high temperature followed by a slow cooling induces the quadruplex formation. These oligonucleotides are very attractive models for further studies on the comparative binding of small molecules on various conformations of nucleic acids.

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## 5-5

### How do factors that influence chromatin compaction affect nucleosome and linker DNA structure?

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The compaction of the chromatin fiber, strongly correlated with gene activity, is known to be influenced by the ionic environment, presence of linker histones, modification of core histones and DNA methylation. We are interested in the effect of these factors on the mononucleosomal level, i.e. on the geometrical path of the linker DNA and on the stability of the nucleosomes. Nucleosomes (NS) were reconstituted from recombinant histones on DNA fragments of different length (150-220 base pairs). Histones were acetylated chemically prior to octamer formation. Then the end to end distance of the double-labeled DNA was determined by fluorescence resonance energy transfer (FRET). The stability of the NS was assessed by UV absorbance and circular dichroism melting. The FRET results suggest that the linker DNA arms do not cross. In the presence of H1 and / or in the presence of 0.1-2 mM Mg2+, they approach each other for all linker lengths. NaCl below150 mM does not change the distances significantly; above this concentration, the DNA is released. Acetylation of the core histones leads to separation of the distant ends of the linker arms. The effect of specific histone acetylation is under study. Melting measurements show that the histone/DNA interaction is insensitive to salt concentration in the measured range; the DNA helix-coil transition is less salt dependent in nucleosomes than for isolated DNA. The presence of histones increases the thermal stability of the DNA. These results are found for all fragment lengths studied and are similar for reconstituted and isolated mononucleosomes.

## 5-6

### Interactions of nucleic acids at charged interfaces

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Interaction of single- and double-stranded DNA with bis-intercalator echinomycin at DNA modified surfaces was studied by electrochemical methods, first of all by measurement of dependence of the differential capacitance C and/or impedance Z of the electrode double layer on potential E (C-E curves) and on frequency (electrochemical impedance spectroscopy-EIS). The measurements were performed at mercury film electrodes (MFE). The surface of glassy carbon electrode (GCE) and pyrolytic graphite electrode (PGE) was covered by a mercury film by galvanisation. The electrode surface was visualised and characterised before and after galvanisation by a metallurgical microscope. The electrochemical studies of echinomycin alone were performed both in the bulk of solution (in situ) and by adsorptive transfer stripping method (AdTS) developed by Palecek. The aim of the study was to find a method for sensing DNA conformation. The dependence of the pseudocapacitance redox peak of echinomycin on concentration and on the a.c. voltage frequency was measured. It was found that with the aid of DNA-echinomycin complex it is possible to distinguish the single-and double-stranded DNA adsorbed at the MFE. The optimum conditions for the sensing of DNA structure were determined. The frequency response of the impedance of MFE double layer in the presence of the DNA- echinomycin complex is sensitive to the DNA conformation as well. This work was supported by Grant Agency of the Academy of Sciences of the Czech Republic A4004901 (F.J.) and S5004107 (V.V.).

## 5-7

### Electrostatic interactions between DNA-oligonucleotides and biased au-electrodes in electrolytes of varying salt concentrations

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We study the bias-induced release of 24mer single stranded (ss) DNA oligonucleotides from Au-electrodes into electrolyte solution of varying salt concentration. Desorption is evidenced by monitoring the photoluminescence (PL) of cyanine-dye labelled DNA, taking advantage of PL-quenching near metal surfaces. The temporal evolution of the observed PL-intensity following a bias-step can be explained by a simple rate model taking into account the optical measurement geometry. Hence, parameters such as the relative desorption efficiency, the release time constant, and the diffusion time constant of released oligonucleotides in aqueous solution can be evaluated. The electrostatic interactions between adsorbed ssDNA and the Au-surface are investigated with respect to (i) a variation of the bias potential applied to the Au-electrode, and (ii) the screening effect of the electrolyte on the electric field extending from the Au-surface into solution. For the latter, the salt concentration of the electrolyte solution is varied systematically from 3 to 1600mM. As expected, we find a decreasing desorption efficiency for increasing salt concentrations, i.e. better screening. However, correlated analysis of optically determined release time constant and electrochemical data suggests that desorption of DNA from the Au-surface occurs mainly during charging of the electrical double layer, on a time scale where electrochemical equilibrium has not yet been established and screening can not be completely described by the Gouy-Chapman-Stern theory. In addition, we observe a decrease of the diffusion time constant of ssDNA in electrolyte solution upon increasing the salt concentration. This is discussed in terms of the DNA's counterion-cloud size and its effect on the DNA's mobility in solution.

## 5-8

### Molecular Dynamics studies of RNA kissing-loop complexes.

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A kissing complex is made of two RNA hairpins non-covalently linked by their complementary loop. Kissing complexes play a key role in a lot of cellular and viral gene regulation processes. Their stability depends on their loop-closing base pairs, and surprisingly in some cases G and A residues is the best combination for a stable dimer. The TAR element of HIV-1, involved in the retro-transcription process, is able to ?kiss? its complement TAR*. The structure of this TAR/TAR* complex has already been published (Chang and Tinoco), and we used it to generate three mutant closing base pair models, UA, CA and GA. Thanks to thermal denaturation experiments, we confirmed that TAR/TAR*(GA) was more stable than the UA and especially the CA mutant. The reasons of these different stabilities were then investigated by molecular dynamics (MD) and we proved that the key parameters to form a stable kissing complex is a loop-closing base pair with a large interglycosidic distance and an efficient stacking. In this case, a Watson-Crick purine-purine base pair like GA seems to be particularly well adapted to this problem. Then we focused on DIS, which is a crucial element for the dimerization process of HIV-1. A crystallographic (Ennifar et al.) and a solution structure (Mujeeb et al.) of DIS/DIS kissing complex were published and differ a lot. These differences were investigated thanks to the MD and we specifically focused on the role and dynamics of the loops? unpaired residues. Finally we proposed a new and simplified dimerization process.

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## 5-9

### The native chromtin structure in single cells: a micro x-ray scattering study

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The organisation of the DNA double helical chain inside the cell nucleus is of outstanding interest for the understanding of the cell division. Several models for the organisation of these nucleosome chains have been derived by different methods. These models vary from a rather disordered amorphous arrangement, over a more zig-zag bended structure of linked symmetrical units to a higher ordered chromatin structure. However, these models where derived used isolated samples, whereas the sample preparation can influence the chromatin structure. In order to investigate the chromatin structure in the native environment of the cell a new approach has to be applied. Since the microfocus beamline ID13 at the ESRF permits an X-ray beam of 5µm the µ-SAXS method can be expanded to the investigation of a single cell. Appling this technique we were able to collect SAXS data of frozen HeLa cells as well as from living cells at room temperature. The scattering data correspond well to previous X-ray data on chromatin suspensions, but show interestingly a texture of the s=0.16 1/nm peak which can be assumed to the nucleosomal core particle scattering. This anisotropic contribution indicates a pre-orientation of the chromatin fibre in the cell nucleus. A further purpose of the µ-SAXS technique is the investigation of single cells during the mitosis. This method takes advantage of the possibility to stall the cell division of HeLa cells upon freezing to 100K. Thus, the cells can be trapped at different reaction steps and investigated using the µ-SAXS approach.

## 5-10

### Low-resolution reconstruction of a 4-way juntion

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The 4-way DNA junction is the recombination intermediate for an important number of site-specific recombinases, and the determination of its global structure is fundamental in understanding the recombination mechanism. We have studied the low-resolution structure of a 4-way junction by using small angle x-ray scattering (SAXS). We have fitted the experimental scattering curves of a 4-way junction with and without Mg2+ with two plausible models, in one the double stranded DNA helices are stacked (stacked-model) and in the other the strands adopt a planar conformation (planar-model). For both models we have performed an extensive search of the parameter space defined by the angle between the strands, their centre-to-centre distance and the DNA kink angle at the crossover site. In addition, we have employed ab-initio low-resolution reconstruction techniques to gain insight into the global conformational changes undergone by the junction upon ion-binding. Our results agree with those of Lilley et al. [1] who reached similar conclusions based on data from fluorescence resonance energy transfer and polyacrylamide gel shift assays, and provide an independent, complementary and more direct way of studying the global low-resolution conformation of DNA molecules and their structural changes. In addition, this same approach can be applied to reconstruct the conformation of DNA in DNA-protein complexes by using small angle neutron scattering where the protein scattering can be contrast matched with ≈43% D2O. [1] Clegg, RM, Murchie, AIH, Zechel, A, Carlberg, C, Diekmann, S and Lilley DMJ. Biochemistry (1992) 31, No. 20.

## 5-11

### Structure information of a high affinity binding site in the hammerhead ribozyme

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We used EPR spectroscopy and DFT methods to study the structure of a single high-affinity Mn2+ binding site in the hammerhead ribozyme. 3-Pulse Electron Spin Echo Envelope Modulation (ESEEM) and Hyperfine Sublevel Correlation (HYSCORE) experiments together revealed that the manganese(II) ion in this binding site is coupled to a single nitrogen with a quadrupole coupling constant k of 0.7 MHz, an asymmetry parameter n of 0.4 and an isotropic hyperfine coupling constant of Aiso(14N) = 2.3 MHz. A possible location for this binding site may be at the G10.1,A9 site also occupied in crystals (Scott et al. Science 1996, 274, 2065). To decide whether the same site is occupied in the crystal and in frozen solution, we performed DFT calculations of the EPR parameters, based on the structure of the Mn2+ site in the crystal. Computations with the BHPW91 density functional in combination with a 9s7p4d basis set for the manganese(II) center and the Iglo-II basis set for all other atoms yielded values of k(14N) = 0.80 MHz, n = 0.32 and Aiso(14N) = 2.7 MHz which fit the experimentally obtained EPR parameters very well. Therefore, we suggest that the binding site found in the crystal and in frozen solution are the same. In addition, we could show by cw and pulsed EPR that Mn2+ is released from this site upon binding of the aminoglycoside antibiotic neomycin B (Kd = 1.2 mM).

## 5-12

### NMR spectroscopy highlight on slow motions in the millisecond time scale, along the nucleic acid backbone

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Knowledge on dynamics of biomolecules appears to be essential in addition to the three dimensional static structure for understanding their biophysical properties. In this respect, nuclear magnetic resonance (NMR) spin relaxation measurements represent an efficient experimental approach for characterizing molecular motions. Phosphorus (31P) NMR spectrocopy can provide important informations about the dynamics of nucleic acids. So far, no relaxation experiment based on inversely detected spectroscopy has been performed because of poor sensitivity, despite a full natural abundance for 31P. Poor sensitivity mostly arises from the existence of multiple homonuclear 1H-1H scalar couplings which are of the same magnitude as the heteronuclear 1H-31P couplings, rendering the 1H-31P magnetization transfer ineffective. In this poster, we propose an inversely detected 31P transverse relaxation rate (R2) measurement experiment. This experiment enables fast measurement of accurate 31P transverse relaxation rates and provides the possibility to detect slow motions mapped by the phosphorus nuclei along the nucleic acid backbone. Dispersion curves show some 31P nuclei experiencing chemical exchange in the millisecond timescale. Under the assumption of a two-state exchange process, the reduced lifetimes of the exchanging sites obtained are in accordance with base-pair lifetime estimates deduced from imino proton exchange.

## *Posters*

*- Nucleic acids and ribosomes -*

## 5-13

**Behaviour of human telomeric DNA in solution studied by photon correlation spectroscopy.**

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The human telomeric DNA has been studied in solution in a wide range concentrations of alkali metal ions by the photon correlation spectroscopy (PCS). The presence of different telomeric structures was observed and their interactions, depending on the ion type, salt and DNA concentration, were analysed. In the presence of monovalent ions a single collective diffusion mode appeared corresponding to intramolecular monomeric quadruplex. The results were confronted with the predictions of the coupled mode theory. The agreement between our data and the theoretical predictions points to the dominant role of electrostatic interactions in solution. The determined effective charge on the monomer molecule was in agreement with the Manning counterion condensation theory. Experimental value of translational diffusion coefficient DT for monomeric quadruplex was obtained. This value is consistent with the bead model calculations, based on the atomic coordinates from the NMR and X ray crystallographic data. The PCS results were supplemented with CD data permitting a determination of the thermodynamical parameters of the temperature phase transition. The results were interpreted in terms of the theory of polyelectrolytes. In the presence of strontium a few collective diffusion modes were observed indicating the structural polymorphism of the sample. One of the modes corresponded to the monomeric quadruplex, while another one to the linear tetramer. The appearance of different aggregates was affirmed. A structural phase transition from monomer to tetramer was observed, the relative weight concentrations of the structures depended on the concentrations of strontium and DNA in the solution were analysed.

## 5-14

**Structural organisation of nucleic acids from sensitive and resistant cancer cells probed by seira**

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We proposed to use a modification of FTIR spectroscopy methods, namely, surface enhanced infrared absorption (SEIRA) for study of structure of DNA from sensitive and resistant cancer cells as well as after anticancer drug applications. Nowadays this problem is a subject of great interest. We have studied the total fraction of DNA isolated from Carcinoma Guerina cells of resistant and sensitive cancer strains of Wistar line rats before and after anticancer therapy. Under the application of doxorubicin to these 2 types of cancers we registered the more changes in SEIRA spectra of DNA from sensitive cancer strain than those for DNA from resistant strain. In the sugar region the SEIRA spectra of DNA looks close to the helix DNA. The intensity of the band at 830 cm-1 decreased by factor 2, numerous states in the 900-700 cm-1 region are now missing, and the intensity of the band at 963 cm-1 (A-form) increased. The increase of the intensities of PO2- bands in 1.5 times for DNA from sensitive and in 1.2 times for DNA from resistant strains have been registered. The last is probably connected with intercalation of doxorubicin in DNA double strand and H-bonding of C14-OH of doxorubicine with P02- of the DNA. Thus, the DNA from resistant cancer strain could be characterised as rigid structure. The structure of sensitive cancer DNA seems to be flexible, and after anticancer application manifests many different structures.

## 5-15

**Relative stabilities of the dimeric structures of Mal and Lai HIV-1 subtypes**

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The dimerization initiation site (DIS) of human immunodeficiency virus type 1 (HIV-1) is a 23 nucleotides hairpin structure. The hairpin loop contains nine nucleotides composed of a self-complementary hexameric sequence flanked by two 5' and one 3' purines. This hexameric sequence and the purines are known to be essential for the dimerization of genomic RNA of HIV-1 (1). This occurs by the formation of a kissing-loop complex consisting of hydrogen bonding between the hexameric sequences of two RNA molecules In this study, we use molecular dynamics simulations, starting from X-ray structures (2) to check the stability of these complexes in solution. The behaviors of the two subtypes are very different: subtype Mal seems to be less stable than subtype Lai. To determine the influence of the self-complementary sequence and of the flanking purines on the kissing-loop complex stability, two mutants were studied starting from subtype Mal. The first one, with U11->C11 and A14->G14 mutations in the hexamer, leads to an hybrid stem-junction Mal/hexamer Lai. In the second one the flanking G9 is mutated with an adenine, leading to a hybrid stem-hexamer Mal/junction Lai. The Mal junction is sufficient to destabilize the Lai kissing-loop complex but the Lai junction does not stabilize the Mal kissing-loop complex. Electrophoresis and melting experiments carried out in parallel leads to similar results than molecular dynamics. (1) J.L. Clever, M.L. Wong, T.G. Parslow (1996), Journal of Virology, 70, 5902-5908 (2) E. Ennifar, P. Walter, B. Ehresmann, C. Ehresmann, P. Dumas (2001), Nature Structural Biology, 8, 1064-1068.

## 5-16

**Conformational transitions in polyA·2polyU, induced by heating and $Cd^{2+}$ ions**

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Differential UV and visible spectroscopy were used to study interactions of $Cd^{2+}$ ions with AMP, UMP, single-stranded polyA and polyU, double-(polyA·polyU (AU)) and triple-(polyA·2polyU (A·2U)) stranded polynucleotides in buffer solution (pH 6-7) with 0,03M $Na^+$ and ($10^{-6}$ - $10^{-2}$)M $Cd^{2+}$ .

UMP absorption spectra do not change up to $3\cdot 10^{-2}$M $Cd^{2+}$ that evidences ion interactions only with the nucleotide phosphate group. N7 and N1 are atoms coordinating $Cd^{2+}$ in AMP and polyA. In the range $1{,}56\cdot 10^{-4}M \leq [Cd^{2+}] < 8\cdot 10^{-4}$M this interaction type results in disordering of polyA helical parts. At $[Cd^{2+}] \geq 8\cdot 10^{-4}$M intramolecular condensation of polyA and polyU into a highly-ordered structure is observed that follows from strong hypochromicity of absorption spectra at $\nu \sim 40000$ $cm^{-1}$. These particles are of high thermal stability and destruct at temperatures ~$80^0$C, forming light-scattering intermolecular aggregates. The chelate complex N7-$Cd^{2+}$-O(P) is the main type of interaction in A·U double helix. $Cd^{2+}$ binding to A·2U phosphates leads to restoration of the helical structure of disordered triplex parts. The character of the heating-induced conformational transition in A·2U changes with the $[Cd^{2+}]$ content: two subsequent transitions A·2U→ A·U+ U→ A+U+U take place in the region $10^{-6}$- $6\cdot 10^{-4}$M; at $[Cd^{2+}] > 6\cdot 10^{-4}$M single-stage unwinding of the triplex into single strands A·2U→A+U+U is observed.

This work was supported by the CRDF, Grant UB2-2442-KH-02.

***Posters***

*- Nucleic acids and ribosmes -*

## 5-17

**Thermodynamic Studies Of DNA With Different Hydration Degree**

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The hydration of biomacromolecules has been a central problem in the molecular biophysics for many than fifty years. However, up to now in spite of a huge quite unique amount of experimental data and of many theoretical approaches we still have many mysteries of the effect of water on biomacromolecule structure, properties and dynamics.
It is known that water plays a crucial role in the determining the way in which DNA forms its native structure and then interacts with other cell components. Then, one of the most important aspect connected with formation of the native structure is restoration of DNA after unfolding during denaturation process.
In the present work the effects of hydration degree on thermodynamic parameters of DNA forming double helix has been determined by the differential scanning calorimetry. DNA samples have been heated with different rates at temperatures between –20 to 120 degrees C at humidities range 2÷98%. It has been shown that the transition of DNA from one conformation into another passes through intermediate states and is accompanied by changes in the thermodynamic values. It has been demonstrated that the whole range of humidities may be divided into three regions where DNA has different molecular structure, for each of these regions thermal peculiarities has been discussed. Special attention has been given to thermal properties of DNA containing only bound water. On the basis of the examined results the problem of DNA structure formation and stability is discussed.

## 5-18

**Physical mechanisms of the interaction with DNA of a new series of actinocin derivates**

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The results of experimental and theoretical investigations of the physical mechanisms of the interaction with DNA of a new series of biologically-active actinocin derivatives with side chains of different length have been reported. The main goal of these studies is to understand the nature of the specificity of interaction between drugs and nucleic acids, taking into account the interaction with water molecules. UV-visible and infrared spectrophotometry, piezogravimetry, differential scanning calorimetry and also the methods of computional analysis have been used to study this problem. It has been found that the melting temperature of DNA in the presence of the actinocin derivatives increases with decrease in the length of the phenoxazone chromophore. Experimental data and results of computer simulation confirm that two types of drug-DNA complexes may exist for the investigated systems: one is intercalation of the planar phenoxazone chromophore into the GC-site of the DNA and the other is binding of the drugs in the minor groove of DNA. A substantial energetic preference is observed for the complexation with DNA of actinocin with two methylen groups in the side chains (ActII). Water molecules occupying bridging positions between the hydration-active centers of the drugs and the DNA provide additional stabilization of the intercalated complexes, being most pronounced for ActII-DNA complexes. These results agree with most biological activity of such drugs (ActII) in a human leukemia cell line.

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## 5-19

**Nucleic Acid Binding Properties of HIV-1 Gag Sequences Containing the Nucleocapsid Domain**

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Retroviral nucleocapsid proteins show nucleic acid binding and chaperone activity. HIV-1 NCp7 (55 a.a.) has two zinc fingers and a sole Trp whose fluorescence is quenched upon binding. It has been proposed that NCp9 (71 a.a.) and NCp15 (123 a.a.), Gag cleavage intermediates carrying an additional Trp, may have properties different from NCp7.
NCp9 and NCp15 showed 2-fold higher affinity in fluorescence titrations with ssDNA, ssRNA and dsDNA, relative to NCp7. Limiting Trp quenching was higher for NCp7 than for NCp9 and NCp15, in support of weak involvement of distal domains in nucleic acid association. Occluded site size increased from 7 nt/NCp7 to 9 nt/NCp9 and 11 nt/NCp15.
We used an optical tweezers instrument to stretch single λ DNA molecules through the helix-coil transition at fixed NC ratio. Whereas NCp15 and NCp9 lowered the transition's cooperativity (an effect correlated with chaperone activity) similarly to NCp7, their significant forces below the DNA contour length suggest stronger dsDNA binding, in agreement with fluorescence titrations. NCp7 zinc finger mutants did not affect DNA stretching, indicating that these domains are critical for altering DNA secondary structure.

## 5-20

**Fluorescence studies of the dimerization initiation site of the HIV-1 genomic RNA.**

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HIV-1 genomic RNA is packaged as a dimer. The Dimerization Initiation Site (DIS) is a conserved stem-loop located in the 5' region of the genomic RNA. The DIS loop contains a self-complementary sequence, which initiates dimerization by forming a kissing-complex. It has been shown that the kissing complex is converted into a more stable complex, called extended duplex. Although crystal structures of these two forms have been solved, the molecular events leading to the mature dimer remain largely unknown. In this context, our goal is to characterize in vitro the molecular mechanisms that govern the transition from the kissing-complex to the extended duplex. By absorbance spectroscopy we obtained the exact conditions leading to the kissing-complex folding. In the same way, by thermal denaturation measures, we observed the cooperative un-folding of the kissing complex as a function of temperature. DIS sequences doubly labeled at 3'- or 5'-extremities by a non-fluorescent quencher and a chromophore constitute a Molecular Beacon. In its closed form, the DIS stem of the hairpin holds the two moieties close together, quenching the fluorescence of the fluorophore. In contrast, any destabilization of the stem restores the fluorescence of the fluorophore. Molecular Beacons constitute thus a powerful tool that would allow by fluorescence spectroscopy a precise investigation of molecular mechanisms that govern the transition from the kissing-complex to the extended duplex. Since dimerization is an essential step for HIV-1 infectivity, this *in vitro* fluorescence studies would contribute to drug design directed against the viral replication.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-1

### Molecular modifications of the pore forming gamma hemolysins from staphylococcus aureus

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Gamma-hemolysins are bi-component toxins which belong, with leukocidins and alpha-hemolysin, to the so called beta-barrel pore-forming toxin family. Either HlgA or HlgC can be combined with the component HlgB to form two active toxins (called AB and CB). In planar lipid bilayers both toxins form cation-selective pores of conductance 110 pS (AB) and 190 pS (CB) and non-linear current-voltage characteristic. Pores are similar in size (radius of 0.9-1.1 nm) and are probably hexamers containing 3 copies of each component. Based on sequence alignment and on the crystallographic similarities between alpha-hemolysin heptameric pore and HlgB, a model of gamma-hemolysin pore has been proposed. It was then possible to investigate the role of some crucial residues that are located in the pore lumen. Modifying the charge of few key aminoacids (HlgA108 and HlgB121) we were able to modulate the electrical pore characteristics (conductance and selectivity). On the other hand, by inserting a reactive cysteine at a position corresponding to the pore vestibule (HlgA141 and HlgB105), we were able to activate the channel by thiol-specific reagents for detecting mercury in solution. These studies have demonstrated the correctness of the elaborated 3D model, and the potential usefulness of such gamma-hemolysins mutants for the construction of biosensors for mercury and cadmium. Sponsored by PAT Fondo Progetti (Project StaWars).

## 6-2

### Site-specific labeling of receptor proteins in living cells to probe protein structure and molecular interactions

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Chemical and biological labeling are fundamental to elucidate the function of proteins. In particular fluorescent probes allow fast, real-time imaging of molecules and molecular assemblies. Here we present a new strategy to label proteins efficiently and reversibly both in vitro and in living cells. An optical probe is bound reversibly to an affinity sequence of a recombinant protein of interest. The feasibility of this approach is demonstrated by labeling in vitro GFP. In live cells distances between a fluorescent ligand bound with high affinity and selectivity to the ionotropic serotonin type 3 receptor and the optical probe were measured via fluorescence resonance energy transfer. Such experiments deliver important structural information, like protein topology and distance separating affinity sequences. The present approach overcomes severe limitations of existing labeling methods, allowing rapid, reversible, site-specific in vivo labeling of proteins by small, non-perturbing molecular probes and thus opens the door to decipher quantitatively the biochemical networks that control and maintain cellular function which is a major goal of proteomics.

## 6-3

### Structure-function relationship of the sodium-d-glucose cotransporter: fluorescence experiments on single tryptophan mutants of the extracellular loop

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The Na dependent glucose cotransporter (SGLT1) facilitates glucose or galactose transport across the apical cell membrane of renal and intestinal cells by the utilization of the inward sodium gradient. The protein consists of 662 amino acid residues having 14 transmembrane domains joined through 13 extracellular or intracellular loops. Phlorizin is known to be a potent inhibitor of the sodium D-glucose cotransporter. Our previous mutagenesis studies have revealed that the C-terminus Loop 13 consisting of 97 amino acids is critically involved in phlorizin binding between amino acid residues 604-610. To study in more detail the interaction between the loop and phlorizin, tryptophan was introduced at position 581, 591, 601, 611, 621 and 630 by site directed mutagenesis. Truncated wild type loop 13 and the mutants were expressed in E.coli and purified to homogeneity. The intrinsic tryptophan fluorescence of each peptide was measured in the absence and upon addition of phlorhizin (25-100 micromole) by using photon-counting Perkin–Elmer Lambda 2 UV/VIS spectrophotometer. The tryptophan mutants at positions 601 and 611 exhibited the highest fluorescence quenching as compared to the other tryptophan mutants. These findings confirm directly that the possible recognition site for phlorizin is located in this part of loop 13.

## 6-4

### Effects of free radicals on bovine serum albumin and human hemoglobin

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Mild oxidation of -SH-containing globular proteins by Ce (IV)-ions in the presence of the spin trap phenyl-tert-butylnitrone (PBN) resulted in strongly immobilized nitroxide free radicals, which evidences the formation of thiyl radicals: protein-SH + Ce(IV) - protein-S + Ce(III) protein-S + PBN - protein-S - PBN In hydroxyl free radical generating system strongly immobilized nitroxide radicals was also detected in these proteins, which implies that the oxidation of a fraction of the thiol groups was also involved in the free radical reaction. The hyperfine splitting constant of the PBN spin adducts was 2Azz=6.406*0.03 mT (n = 4. This suggests that the PBN spin adduct molecules located on the single cysteine residue of the proteins. The DSC measurements were evaluated according to the two-state kinetic model. The activation energies were Ea = 280* 4 kJ/mol (native BSA) and 230*6 kJ/mol (Ce (IV)-NTA treated BSA), respectively. The narrow peak (Tm = 4.1C, the width of the melting curve at half-height) detected at (57.0* 1.0) C (n=5) in the absence of Ce (IV)-NTA complex showed a large broadening (Tm = 14.6 C) after Ce (IV)-NTA treatment. The results support the view that site-specific interaction of SH-containing proteins with free radicals is able to modify the internal dynamics of proteins and affect the conformation of large molecules. (This work was supported by grants ETT 370/2000, FKFP 0387/2000 and OTKA CO-123, CO-272).

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-5

**Spectroscopic differentiation of the substeps involved in Sr2+ or Ca2+ dissociation from serca1a**.

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Lys515 in the nucleotide binding domain in SERCA1a, the membranous ATPase responsible for Ca2+ transport into sarcoplasmic reticulum, was specifically labelled with fluorescein isothiocyanate (FITC), and the overall rise in FITC fluorescence previously shown, in steady-state fluorescence experiments, to accompany the dissociation of the two Sr2+ or Ca2+ ions initially bound to labelled FITC-ATPase, was now studied in time-resolved stopped-flow experiments. Simultaneously, we also measured the kinetics of Ca2+dissociation per se, by using quin2, a Ca2+-sensitive fluorophore, as the chelator. We found that the rise in FITC fluorescence, triggered by the addition of a fast Ca2+ chelator, was preceded by a prominent lag period. This lag period revealed by FITC which is bound to a cytosolic domain far from the transmembrane Ca2+ binding sites, suggests that conformational rearrangement of the ATPase does not occur immediately upon dissociation of the first of the two Ca2+ (or Sr2+) ions initially bound, and probably not even upon dissociation of the second ion, but only occurs during conformational reorganization of the ion-free ATPase. The latter possibility is also suggested by the fact that when FITC fluorescence is monitored during Ca2+ or Sr2+ binding to FITC-ATPase that has been initially depleted from divalent cations, the changes in FITC fluorescence now occur at the same rate with the two ions (with no lag, and of course in the opposite direction). The suggested conformational equilibrium of ion-free ATPase probably contributes significantly to the cooperativity with which Ca2+ and Sr2+ can bind to their transport sites.

## 6-6

**Ligand migration in myoglobin under xenon pressure**

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In recent years, cryo crystallography of photolyzed intermediates and time-resolved crystallographic studies of carbononoxy-Myoglobin (MbCO) have revealed that the photo dissociated CO molecule may reside in one or the other of several docking sites. The latter however differ among authors depending at which particular temperature these difficult experiments could be performed. Around room temperature CO was found to occupy a hydrophobic cavity known to bind Xenon and, indeed, it has been reported that the kinetics of geminate rebinding in oxy-Myoglobin (MbO2) are affected by Xenon. In Mb, crystallography has identified long ago four such cavities that are able to bind non covalently a Xenon atom. Thus, kinetic competition experiments involving ligand rebinding in presence of Xenon provides a means for probing ligand migration. To get a more general insight into the role played by these cavities in ligand migration, systematic kinetic measurements over a wide range of temperature were needed. We have performed laser-photolysis experiments with MbO2 and MbCO in water/glycerol solution under moderate Xenon pressure (1-20 bars) and over the temperature range 77 K-300 K. Using the Maximum Entropy Method to convert the kinetics into rate distributions and performing a global analysis of the rate spectra, we were able to observe new kinetic features indicating that in Mb the ligand follows at least two different migration and escape pathways that smoothly interchange with temperature. This approach provides a continuous view with temperature of the kinetic consequences of blocking the access of the ligand to the hydrophobic cavities of a protein.

## 6-7

**Structure and orientation of bacteriorhodopsin and lipid in the oriented purple membrane**

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Bacteriorhodopsin (bR) is one of the most extensively studied membrane proteins as a prototype of G-protein coupled receptors. BR forms protein-lipid complex known as purple membrane (PM) in which the main lipid is phosphatidylglycerophosphate methyl ester (PGP-Me) [1] and PM film on the glass plates is known to orient well [2]. In the 31P NMR spectra two signals assigned to a alpha- and a gamma-phosphate group of PGP-Me were observed. Hydration of the PM is determined to induce better alignment of the lipid head groups. By decreasing temperature reversible spectral changes were observed between –35 and –40 deg C and that is similar to the changes seen for phase transition. [15N]Met-labelled bR, where all 9 residues in bR are labelled, has been used to determine the orientational and conformational constrains of helices and loops of bR in the PM. The polarization inversion spin exchange at magic angle (PISEMA) spectra and 1H-15N correlation spectra (HETCOR) showed complimentary reasonable resolution on the signals from helices and loops, respectively. The helix region of the experimental spectra were deconvoluted to obtain tilt and rotation angle of the helices. We estimated the helix B in the PM film tilts around 5 deg from the membrane normal, which is identical to the highest resolution crystalline structure, 1C3W [3]. 1. Kate, K., et al (1982) Methods Enzymol. 88, 98. 2. Ulrich, A., et al. (1992) Biochemistry 31, 10390. 3. Luecke, H. et al. (1999) J. Mol. Biol. 291, 899.

## 6-8

**Real-time detection of time:resolved dynamics changes of the cytoplasmic loop domain of rhodopsins during function**

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The conformation of the cytoplasmic loops of bacteriorhodopsin and bovine rhodopsin were assessed by a combination of time-resolved fluorescence depolarization and site-directed fluorescence labeling. The fluorescence anisotropy decays were measured employing a tunable Ti:sapphire laser/microchannel plate based single-photon counting apparatus with picosecond time resolution. This method allows to measure the diffusional dynamics and the motional freedom of the interhelical segments directly on the nanosecond time scale. The most recent results regarding the transmembrane proteins rhodopsin and bacteriorhodopsin (bR) are as follows. We were able to identify two mechanisms of loop conformational changes in the functionally intact proteins bR and bovine rhodopsin: we found a surface potential change-based switch between two conformational states of the EF-loop of bR and a particularly striking pH-dependent conformational change of the fourth loop on the cytoplasmic surface of rhodopsin. The functional significance of the detected mechanism of conformational changes can be determined by real-time monitoring of the time-resolved anisotropy decay of the fluorescent dye during the time course of the photoreaction of bR and rhodopsin. A first example is the detection of the time-resolved motional freedom and dynamics changes of the CD-loop of bR during its photocycle with second time-resolution. At least three different conformational states of the CD-loop during the M-intermediate and its decay were resolved. New insights into dynamics-function relations regarding the proton uptake mechanism and protein-protein interactions on the cytoplasmic surface of 7-helix transmembrane proteins were found.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-9

### Nucleotide binding kinetics of Na,K-ATPase: effect of cations

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Na,K-ATPase (EC 3.6.3.9), one of the P-type ATPases, is responsible for transport of 3Na+ and 2K+ against their electrochemical gradients at the expense of the energy of 1 ATP-molecule. The widely accepted hypothesis, describing the mechanism of energy transduction from the hydrolytic site to the cation-transporting sites, involves transition between two major protein conformations - E1 and E2. This concept, based on the experimental evidence, requires an interplay between the hydrolytic (nucleotide binding) site and the cation binding (transport) sites. Na-occupation of the transport sites enables high affinity nucleotide binding, inducing the protein conformation defined as E1. Low affinity nucleotide binding to the E2-conformation of the enzyme (the K+-occluded state) promotes K+-release from the cation binding sites. The enzyme is highly selective: despite only a small difference in ionic radii of Na+ and K+ (0.095 nm vs. 0.133 nm) it is able to distinguish between those ligands. It was shown, however, that other cations (Tris, imidazol, choline chloride, N-methyl glucamine) have a Na+-like influence on a number of partial reactions of Na,K-ATPase, including nucleotide binding. The question is if these cations implement their effect through binding to the specific transport sites. To elucidate the mechanism of the ion effect we studied the cation dependence of the enzyme affinity for ADP. We compared the efficiency of different cations to induce high affinity nucleotide binding (E1-conformation) with their ability to compete with K+. The techniques employed in the present study included both transient and equilibrium measurements of nucleotide binding.

## 6-10

### Histone H1 and acidic phospholipids form supramolecular aggregates in the cell membrane

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Histone H1 is involved in the formation of high order chromatin structures and in the modulation of gene expression. However, this protein performs also extracellular functions. We have demonstrated that histone H1 induced apoptosis of cancer cells. Further, we could show that this is due to its interaction with acidic phospholipids in cell membranes. Histone H1 adopts a random structure in aqueous solution and in the presence of neutral phosphatidylcholine bilayers. Histone H1 binds preferentially to liposomes containing anionic phospholipids. Interestingly, a pronounced increase in its a-helicity was evident in trifluoroethanol and in membranes containing phosphatidylserine (PS), an acidic phospholipids. Also the macroscopic consequences of the binding of H1 to membranes were enhanced significantly by PS. More specifically, histone H1 caused strong aggregation, fusion, and rupture of liposomes containing PS whereas neutral membranes remained intact. Vesiculation of zwitterionic giant liposomes by histone H1 was observed only when the membrane was exposed to high amounts of the protein. Membrane bound histone H1 aggregated on the surface of giant liposomes containing PS. Subsequently, the aggregates transferred from the outer leaflet of the bilayer into the internal cavity of the giant liposome. Histone H1 and acidic phospholipids form supramolecular aggregates in the cell membrane and increase permeability of the membrane, finally leading to apoptosis and cell death.

## 6-11

### Hydrophobic pockets at membrane interface : an original mechanism for membrane protein interactions

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The effect of partial digestion by trypsin and GluC proteases on the association of two small transmembrane polypeptides (alpha and beta) of the photosynthetic core complexes (LH1) from Rhodospirillum rubrum was studied. Cleavage sites were identified by mass spectrometry. Trypsin and GluC proteases digestion result in the cleavage on N-terminal ends of the first three amino acids from the alpha polypeptide and of the sixteen first amino acids from the beta polypeptide, respectively. The influence of the protease attack on the secondary structure of polypeptides was controlled by Fourier-Transform Infrared Spectroscopy (FTIR). None of these treatments induced large change in the secondary structure. However, the enthalpy variation accompanying dimer formation was dramatically reduced, by 30 and 80 % upon trypsin and GluC treatment, respectively. Electrostatic interactions do not seem to play a role in this reaction. These results show that the amino-terminal extensions of these polypeptides play an important role in promoting the dimerisation of the two membrane polypeptides, through the formation of hydrophobic pockets at the membrane interface. Using the close homology between the polypeptides of Rsp. rubrum LH1 and that of Rsp. molischianum LH2, whose structure is known, a structural model implicating the hydrophobic pockets is proposed.

## 6-12

### Characterisation of the c-terminal domain of the sm-like protein HFq from E. coli

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The HFq polypeptide of E. coli is a nucleic acid binding protein involved in the synthesis of many proteins. HFq particularly affects the translation and the stability of several RNA. Utilisation of fold recognition methods allowed us to detect a relationship between Hfq and the Sm topology. This topology was further validated by a series of biophysical studies and the Hfq structure was modelled on a Sm protein. Hfq forms a ring shaped hexamer with a continuous beta sheet. Because our bioinformatic study suggest a large number of alternative conformations for the C-terminal region, we have determined whether the last 19 C-terminal residues are necessary for protein function using a proteolised protein. We find that the truncated protein is fully able to bind RNA (Kd of 120 pM vs 50 pM for full length HFq). This result suggests that the functional core of the protein resides in residues 1-80, namely the Sm domain. By using equilibrium unfolding studies, we find that full length Hfq is 1.8 kcal mol-1 more stable than truncated protein. Comparison of average images from electron microscopy for both proteins indicates a structural rearrangement between the subunits. This conformational change is coupled to a reduction of the b-strand content by seven aa (determined by FTIR). On the basis of all these results, we propose that after proteolysis, residues 66-83 folds back against the last beta-strand and modify the interface between two consecutive monomers. The origin of the C-terminus domain will be discussed at this conference.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-13

### Behaviour of water confined by biological macromolecules and its relation to protein dynamics

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The study of water confined by biological entities is pertinent to the question as to which degree the dynamics of proteins and their direct environment are coupled. We have used two complementary techniques, neutron scattering and X-ray crystallography, to study the behaviour of water confined in protein crystals and in stacks of biological membranes and to address the issue of dynamical coupling of proteins and their environment. A neutron diffraction study of flash-cooled amorphous water confined in stacks of purple membranes showed that, upon heating to 200K, it adopts liquid-like behaviour. The dynamical transition on the nanosecond time scale of the purple membrane itself takes place at ~ 260 K, as determined by incoherent elastic neutron scattering. Thus, the glass transition of inter-membrane water takes place at least 60 K below the nanosecond dynamical transition of the membrane. In a second approach, X-ray crystallography was employed to study the temperature-dependent solvent behaviour in flash-cooled protein crystals in general and the relation to protein flexibility in particular. Structural changes that arise in the enzyme acetylcholinesterase upon X-ray irradiation were used to assay protein flexibility at two temperatures – below (100 K) and above (155 K) the glass transition temperature of the crystal solvent. Conformational changes were observed in the active site at 155 K but not at 100 K. This showed that at the solvent glass transition temperature the protein has acquired, at least locally, sufficient conformational flexibility to adapt to irradiation-induced alterations in the conformational energy landscape. Combing these results, we conclude, that dynamical changes in a protein's environment can affect protein flexibility locally even though the global protein dynamics might remain unaffected.

## 6-14

### Influence of proline residues in transmembrane helix packing

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The monomer–dimer equilibrium of the glycophorin A (GpA) transmembrane (TM) fragment has been used as a model system to study helix–helix packing in membrane-mimetic environments. In particular we have focused our experiments in the role that proline residues play in different positions of the interacting helices. This amino acid is considered a potent 'helix-breaker' in soluble proteins being underrepresented in this kind of structures. However it is found to be widely distributed in the helical TM domains of many membrane proteins, suggesting a different role in this class of proteins. It has been proposed that prolines occurring in TM domains may have more than one structural and/or dynamic roles depending on the local and heterogenic environment imposed by the phospholipid bilayer. In this context we have used the GpA TM segment as a natural scaffold to screen the effect of the substitution of all its alfa-helical residues by prolines, in an attempt to understand the conformational contribution of this residue in helix packing depending on its position in the TM segment; i.e. near the lipid/water interface or embedded in the hydrophobic core, at the fatty acids hydrocarbon region. Our results suggest that prolines are well tolerated near the interfacial region of the TM fragment, allowing a significant degree of dimerization in the GpA system and in synthetic TM peptides. Computational modeling and molecular approaches in biological membranes suggest a partially extended structure in mutants where proline is located in the first alfa-helical turn of the TM fragment.

## 6-15

### Structure and dynamics of proteins encapsulated in silica hydrogels by trp phosphorescence

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The intrinsic Trp phosphorescence of proteins encapsulated in silica hydrogels demonstrates the usefulness of the delayed emission for examining potential perturbations of protein structure-dynamics by the silica matrix. Phosphorescence measurements were conducted both in low temperature (140 K) glasses and at ambient temperature on the proteins apo- and Cd-azurin, alkaline phosphatase and liver alcohol dehydrogenase together with the complexes latter with coenzyme analogs ADPR and H2NADH. While spectral shifts and broadening indicate that alterations of the Trp microenvironment are more marked on superficial regions of the macromolecule the decay kinetics of deeply buried chromophores show that the internal flexibility of the polypeptide in 2 out of 3 cases is significantly affected by silica entrapment. Both the intrinsic lifetime and the bimolecular acrylamide quenching constant confirm that, relative to the aqueous solution, in hydrogels the globular fold is more rigid with azurin, looser with alcohol dehydrogenase and substantially unaltered with alkaline phosphatase. It was also noted that large amplitude structural fluctuations, as those involved in coenzyme binding to alcohol dehydrogenase or thermally activated in alkaline phosphatase, were not restricted by gelation. Common features of silica entrapped proteins are pronounced conformational heterogeneity and immobilization of rotational motions of the macromolecule in the long time scale of seconds.

## 6-16

### Perturbation of protein tertiary structure in frozen solutions revealed by 1,8-anilino-naphtalene-sulfonate fluorescence

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We have examined the possibility to appraise freeze-induced alterations of protein structure by the extent of ANS binding to proteins in ice. The results of this study, conducted with the monomeric protein azurin, indicate that ice formation leads to a remarkable enhancement and blue shift of ANS fluorescence a finding that is consistent with binding of ANS to the protein. The extent of the process is significantly modulated by experimental conditions that affect the stability of the native fold and is totally abolished when the protein is denaturated in guanidinium-chloride. It is shown that both Cd2+ binding to the apoprotein and the addition of stabilizing agents (glycerol) prevent ANS binding to the protein whereas low concentrations of a soft denaturing agent, such as potassium thiocyanate, further enhances the ANS emission. Indications of partial unfolding upon freezing have been inferred from the shortening of the phosphorescence lifetime (tau) of Trp residues buried in the rigid core of the macromolecule (G.B. Strambini and E. Gabellieri (1996), Biophys. J. 70, 971-976). The correlation between ANS binding and t confirms that the perturbation of tertiary structure inferred by tau are generalized over the entire macromolecule.

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*- Protein structure dynamics and functions (I) -*

## 6-17

### Rapid substrate-induced charge movements of the gaba transporter gat1

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During synaptic transmission, active transport mediated by high affinity neurotransmitter transporters plays an important role in regulating the synaptic neurotransmitter concentration as a function of time. In particular, GABA reuptake is performed by specific GABA transporters and involves the co-transport of two sodium ions and one chloride ion per GABA molecule transported. We investigated the individual reaction steps of the transporting cycle, such as GABA binding and ionic movements triggered by this binding. The laser-pulse photolysis technique together with caged GABA was used to generate GABA concentration jumps within less than 100 microseconds. Simultaneous recording of transmembrane currents from HEK293 cells transiently transfected with GAT1 has shown multiple charge movements that can be separated along the time axis. In the forward transporting mode the deactivation of the transient component of the GABA-induced coupled transport current exhibits three components, one outwardly and two inwardly directed, with distinct voltage-dependence, and that take place on different time scales ranging from the submillisecond to the millisecond range (t1= 0.3ms, t2= 2.8ms and t3=11.4ms, respectively) at saturating GABA concentration. In the homo-exchange transporting mode two components (the fast outwardly directed and one of the inwardly directed) are present. These experiments show that the detected electrogenic reaction steps may be associated to the GABA translocating half-cycle of the transporter, in contradiction to previous studies that showed no charge movements associated to these reactions. Chloride-substitution experiments produced no significant modification of the detected currents. This work was supported by the Portuguese Foundation for Science and Technology.

## 6-18

### Electrostatic effects in recognition of m-rna-cap analogs by translation initiation factor eIF4e

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The key participant in the regulation of translation in eukaryotic cells is the eIF4E protein, which specifically recognizes the 7-methyl-GpppN (G is guanosine, p is a phosphate group, and N is any nucleoside) of the 5'-terminus of mRNA during initiation of translation. Besides stacking of the alkylated guanosine base between the side chains of two conserved tryptophans (Trp56 and Trp102 in the murine eIF4E), which is recognized as the most important factor, there are a number of electrostatic interactions responsible for eIF4E-cap recognition and for dependence of the complex stability on the environmental parameters, pH and ionic strength of solution. We investigated these electrostatic aspects by kinetic measurements in a stopped-flow spectrofluorometer and computer simulations based on the Poisson-Boltzmann model of solute-solvent systems, a procedure for prediction of protonation equilibria in proteins, and a Brownian dynamics algorithm. Investigation of the pH-dependence of binding of analogues of the 5'-mRNA cap to the eIF4E indicated that there are two titratable groups in the binding site of the protein and two titratable groups on the ligands directly involved in the binding, in addition to the stacking interactions mentioned above. In particular, it is concluded that binding of both forms of the cap analogs regarding protonation at the N1 position of the guanine ring is efficient, and the shift to a predominantly protonated form of the ring takes place after formation of the complex. We observed also a semiquantitative agreement between experimental and computed eIF4E-cap bimolecular association rate constants.

## 6-19

### Crystal structures of a wild-type cre recombinase-lox p synaptic complex

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Cre recombinase catalyses the site-specific recombination of DNA containing loxP sites made up of an 8-base pair spacer and two 13-base pair inverted repeats. Cre requires no accessory proteins and mediates all steps in site-specific recombination from synapse formation, DNA cleavage, strand exchange, religation, to Holliday junction isomerization and resolution. We have determined the crystal structure of a wild-type Cre recombinase-loxP synaptic complex. In contrast to previously determined structures, it contains a full tetrameric complex in the asymmetric unit, showing that the antiparallel arrangement of the loxP sites is an intrinsic property of the Cre-loxP recombination synapse. The conformation of the spacer is different to that seen in complexes containing loxA and loxS sites (symetrized versions of the loxP site). A kink next to the scissile phosphate in the top strand leads to unstacking of the TpG step and a widening of the minor groove. Since this side of the spacer is interacting with a cleavage-competent Cre subunit, our structure suggests that the first cleavage occurs at the ApT steps in the top strands at the left hand side of the spacer, and furthermore that the Cre-induced kink next to the scissile phosphodiester activates the DNA for cleavage at this position and facilitates strand transfer. Furthermore, we were also able to solve the structure of a 3'-phosphotyrosyl covalent intermediate resulting from the first DNA strand cleavage. The cleavage, as well as the covalent bound between a tyrosine and the scissile phosphate confirms that the top strand is cleaved first.

## 6-20

### Effects of cavity forming mutations on the internal dynamics of azurin

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The effects of cavities on internal dynamics of azurin were assessed by both the intrinsic phosphorescence lifetime of the deeply buried Trp residue, which reports on the local structure about the triplet probe, and the bimolecular acrylamide phosphorescence quenching rate constant that is a measure of the average acrylamide diffusion coefficient through the macromolecule. Cavities of 40 and 90 angstrom cubici were introduced in the hydrophobic core of azurin, surrounding W48, through the substitution of bulky I7 and Phe110 with Ser, respectively. X-ray structures confirm that the two engineered azurins maintain a fold that, except for small changes in the immediate proximity of the mutation, is identical to the native protein. Phosphorescence measurements demonstrate that with the creation of a cavity the conformational freedom of the indole ring may either increase (I7S) or decrease (F110S) but the rate of acrylamide migration is dramatically enhanced by 3-4 orders of magnitude. These findings emphasize that large amplitude structural fluctuations of the native fold, that seem to be correlated with the instability of the globular structure, rely critically on the internal free volume.

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## 6-21

### Is the pore-forming toxin pneumolysin structurally homologous to perfringolysin o? A solution study

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Pneumolysin (PLY) is an important protein virulence factor of the human bacterial pathogen Streptococcus pneumoniae. PLY could be a candidate for inclusion in a new anti-streptococcal vaccine. There is as yet no high resolution structure for PLY therefore current work on PLY molecular mechanisms is guided by a homology model built on the basis of the perfringolysin O (PFO) crystal structure. PFO, a virulence factor of the organism Clostridium perfringens, has almost the same monomer molecular mass as PLY and shares 48% sequence identity and 60% sequence similarity with PLY. Here we report a comparative low-resolution structural study on PLY and PFO using analytical ultracentrifugation (AUC) and small-angle x-ray scattering (SAXS) to prove (or disprove) a shape similarity for these two bacterial toxins in solution. The size distribution method of sedimentation velocity data treatment was used to show that both proteins in solution are mostly monodisperse but a significant difference in the values of sedimentation coefficient (s) was observed for PLY (3.56 S) and PFO (5 S). This was confirmed by mass determination via the sedimentation equilibrium technique. Thus PLY is a monomer in solution while PFO is a tight dimer. Ab initio dummy atom and dummy residue models for PFO and PLY were restored from the distance distribution function derived from experimental SAXS curves. In solution PLY is an L-shaped, two domain elongated particle; the PFO dimer is an elongated particle not consistent with a simple dimeric model constructed from the known high resolution structure.

## 6-22

### Analysis of the structure and stability of the regulatory protein StyR.

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StyR is a regulatory protein coded by Pseudomonas sp.Y2 involved in the biotransformation of styrene into phenylacetate. StyR is the acceptor of the phosphoryl group from the StyS sensor, protein which detects the presence of styrene in the external environment. Phosphorylated StyR is therefore able to activate the catabolic genes of the pathway. From sequence comparisons with similar regulatory proteins, StyR may be structured in: a.-N-terminal domain: receiver of the phosphate group; b.-Q-linker and c.-C-terminal domain, that binds to the DNA promotor sequence of the catabolic operon. We have modeled the three-dimensional structure of StyR and studied the thermal, pH and chemical stability of the StyR protein and its isolated N-terminal domain as well as the effect of phosphorylation on these parameters. Our results suggest that both domains are structurally independent and that the isolated N-terminal domain achieves a native conformation in absence of the C-terminal domain.

## 6-23

### Dynamic studies of the EGF-like domains of thrombomodulin

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Thrombomodulin (TM) an endothelial cell surface glycoprotein forms a 1:1 complex with Thrombin. This protein-protein interaction causes thrombin to change from a procoagulant protease, to an anticoagulant protein C activating protease. The 81 residue TM fragment composed of the 4th and 5th EGF-like domains (TMEGF45) is the smallest active fragment and has recently been solved by NMR. Interestingly the solution structure showed that the 5th domain was less well ordered compared to the structure of the 5th domain alone, whereas the 4th domain showed no particular differences. The solution structure also showed that M388 residue located in the linker region between the 4th and the 5th domains inserts into the core of the 5th domain. In order to ascertain whether the lack of order in the 5th domain is due interactions with the 4th domain, we have produced each EGF-like domain separately. The domains were produced in Pichia pastoris and so were glycosylated allowing us to also determine the differences in backbone order due to glycosylation. To test the importance of the linker region as a bridge of communication in between the two domains we have carried out studies on different methionine mutants that alter the activity of TM. Results from 15-N relaxation experiments performed on the individual fragments and the mutants are compared to results from the wild type two-domain fragment will be presented.

## 6-24

### Emission spectroscopy of complex formation between e. Coli purine nucleoside phosphorylase (pnp) and identified tautomeric species resolves ambiguitie

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Emission (fluorescence and phosphorescence) spectroscopy of complex formation between E. coli PNP and specific inhibitors formycin A (FA) and N(6)-methylformycin A (m6FA) demonstrated preferential binding of identified tautomeric species, and resolved ambiguities found in crystallographic studies, where tautomeric forms were indistinguishable. Analysis of the static and time-resolved emission, excitation and absorption spectra of enzyme-ligand mixtures pointed to fluorescence resonance energy transfer from protein tyrosine residue(s) to FA and m6FA base moieties, as a major mechanism of protein fluorescence quenching. Effect of enzyme-inhibitor interactions on nucleoside excitation and emission spectra of fluorescence and phosphorescence revealed shifts in tautomeric equilibria of the bound ligands. With FA, which exists predominantly as the N(1)-H tautomer in solution, the proton N(1)-H is shifted to N(2), independently of the presence of orthophosphate (Pi, substrate). Complex formation with m6FA led to a shift of the amino-imino tautomeric equilibrium in favour of the imino species in the absence of Pi; by contrast, in the presence of Pi, the equilibrium was shifted in favour of the amino species, accompanied by a higher affinity for the enzyme. These provide an excellent example of the complementation of solution and crystallographic studies.

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## 6-25

**Folding kinetics of 2-state proteins: effect of circularization, permutation, and crosslinks**

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Protein folding kinetics has recently been probed by clever experiments using circular permutants and other topological mutations. A circular permutant is created from a wildtype protein by covalently linking together the chain ends and cleaving elsewhere in the chain. An interesting puzzle is why circular permutation causes no apparent change in the folding mechanism of CI2 [Otzen and Fersht; Biochemistry 37, 8139 (1998)], but dramatic changes in the folding mechanisms of S6 [Lindberg, Tangrot, and Oliveberg; Nature Struct. Biol. 9, 818 (2001)] and of an SH3 domain [Viguera, Serrano, and Wilmanns; Nature Struct. Biol. 3, 874 (1996)] as determined by Phi-value experiments. Here we use a computational model to predict the folding routes of topological variants, based on a measure (ECO) of the chain entropy loss at each folding step. The model predicts that proteins fold by "zipping up" in a sequence of small-loop- closure events, depending on the native chain fold. The predictions are consistent with the experiments, leading to insights into the folding routes and into the meaning of Phi-values in general. We find that Phi-values do not describe time sequences of folding events, or positions along a single reaction coordinate, as is expected from the standard Bronsted-type analysis; rather, Phi reflects only the degree of rate control. For example, circularization and the circular permutation P40-41 of CI2 is predicted to reverse the time sequence of beta1-beta4 and beta2-beta3 formation, without changing the diffuse Phi-value distribution, while the P13-14 mutant of S6 switches the rate-limiting step from the beta1-beta4 formation to beta1-beta3, changing the Phi-value distribution from diffuse to strongly polarized. As a test of the model, we propose mutations that should reverse these outcomes.

## 6-26

**Highly heterogeneous micro-unfolding of fibrous collagen studied by IR spectroscopy and isotopic exchange**

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Collagen molecules self-assemble into fibrils upon increasing temperature. Temperature-induced origin of assembly suggests increasing entropy (number of degrees of freedom) upon fibril formation. The sources of entropy increase are not well understood. This study addresses possibility of contribution of peptide backbone to the entropy increase by using FT-IR spectroscopy/microscopy of collagen (type I). We studied exchange rate of H/D isotopic exchange of glycines that form interchain hydrogen bonds and determine triple helical structure of collagen. This exchange rate is related to helix reversible micro-unfolding. We found fractions of residues have highly different rates: 15% of glycines of fibrous collagen micro-unfolds ones per day, while the majority of glycines unfolds on the millisecond time scale. Upon fibril formation, conformation of backbone region(s) containing the fast fraction of glycines becomes more disordered and micro-unfolding rate of these regions increases, as indicated by our data on selectively H/D substituted glycines. Apparently, loosening rather than partial melting of some backbone regions occurs during fibril formation, because we observed no substantial exchange of both fast and slow glycines (melting would result in exposure to solvent and complete exchange). We conclude that loosening of extended regions of collagen backbone (containing about 50% of glycines) may be a source of the entropy increase.

## 6-27

**Calorimetric study of thermal destruction and subsequent restoration of native structure in humid globular proteins and their concentrated water solut**

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Our calorimetric studies of thermal properties of the globular protein-water systems in a wide range of water content have shown that the renaturation process is possible not only in diluted solutions as it was considered previously. As we have established earlier the humid proteins can exist in glassy state and the heating causes the transition from the glassy state into the rubber-like one. As a sequence the denaturation of partly dehydrated proteins takes place on the background of this transition and the it is irreversible under such conditions. However, it was found that thermally denatured humid proteins (ribonuclease, lysozyme, and myoglobin) are able to restore their native structure at subsequent dissolving in water. We assume that renaturation in this case is due to the formation during thermal denaturation of rather stable intermediates with structure intermediate between that of native and denatured protein. These intermediates have short lifetime at high temperatures, however can last for unlimited time at room temperature being in the glassy state. In the case of the concentrated solutions the ability to restoration of protein native structure as it was found is also connected with the formation during thermal denaturation the intermediates which are similar the state of the melted globule in dilute solution. These intermediates are fixed by the thermoreversible melting gel network forming under the certain protein environmental conditions can exist for a long time up to the gel melting. The quenching of the sample immediately after the melting of gel prevents gel formation and allows the conditions for renaturation.

## 6-28

**Engineered bacterial protein displaying new binding specificity**

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Neocarzinostatin (NCS) belongs to a family of antibiotic chromophore proteins of bacterial origin, displaying a strong structural similarity with the immunoglobulin domain. The antibiotic activity of this protein is provided by an enediyne chromophore which produces damage to DNA by radical reaction. The role of apo-NCS is to carry and protect the chromophore against hydrolysis. The structural similarity between immunoglobulin and NCS suggests that the loop equivalent to the immunoglobulin CDR can be modified to confer new binding specificity to NCS. To test this hypothesis, we have transferred the CDR3 of an immunoglobulin from an anti-lysozyme camel antibody to the corresponding loop of neocarzinostatin. Studies of the structure, stability and lysozyme affinity of the NCS-CDR3 have been performed. There revealed that the engineered NCS-CDR3 presents a structure close to the WT-NCS, is very stable and is efficiently produced. By ELISA as well as ITC and Biacore measurements we have demonstrated that the new NCS-CDR3 is able to bind lysozyme specifically with a Kd of about 30microM. These results show that NCS is novel scaffold that can be used for grafting new affinity. Compared to other protein scaffolds, NCS has the advantage of presenting two regions that can be engineered : the loop equivalent to the immunoglobulin CDR3 and the natural chromophore binding crevice. This could be particularly relevant for the development of drug targeting for the NCS/chromophore complex is a powerful antitumoral agent currently used in therapeutical applications.

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## 6-29

### NMR investigation of the folding of thymosin beta4 upon binding to actin-G

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Thymosin beta4 (TB4) is a major actin-sequestring protein 1, yet the structural basis for its biological function is still unknown. It was previously shown that the 43 aa peptide was mostly unstructured in solution, except for a short helical region 2. Mutagenesis studies indicated that the alpha-helix was required for bonding to actin. In order to better understand how TB4 prevents monomeric actin assemblies in filaments, we undertook a complete structural study of TB4 bound to actin. We show that TB4 and G-actin form a 1-1 complex in slow exchange at the NMR time scale, and that the whole backbone of TB4 is involved in the interaction. In spite of the large size (43kD) and the limited reachable concentration and stability of the complex, good quality spectra were obtained on a 800MHz spectrometer. The large scattering of {1HN-15N} resonances on the 15N-HSQC spectrum of the complex is in favor of a folding of TB4 upon binding. A selective labeling strategy allowed us to assign the {1HN-15N} resonances of TB4 bound to G-actin. This enabled the analysis of (1H->15N) heteronuclear nOes, which shows a high restriction of mobility in the ps-ns time scale upon binding, compatible with a high structuration of the protein upon binding. A complete structural analysis is currently under progress using {15N, 13C, 2H}-T?4.

## 6-30

### Structural and functional characterization of annexin a13b

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Annexins constitute a superfamily of calcium-binding proteins characterized by their ability to bind to phospholipids in a calcium-dependent manner through a homologous protein core. Annexin-A13, an epithelial-specific member, can be expressed as two splice variants: A13a and A13b (with a 41-residue insertion in the N-terminus). Recombinant annexin-A13b was produced and purified taking advantage of its ability to reversibly interact with phosphatidylserine (PS) rich vesicles; its identity was confirmed by mass spectrometry and N-terminal sequencing. We have carried out exhaustive circular dichroism and fluorescence emission spectroscopic analyses. Calcium binding a) induces a slight modification in the far-UV CD spectrum, b) raises the Tm from 44 to 54 C, and c) exposes the unique Trp residue, located in the 3AB calcium-binding loop, to the solvent. A decrease in the pH is also characterized by a similar conformational change in the Trp environment: while almost no variation is detected in the CD spectra at mild acidic pH, an exposure of the Trp residue is observed by fluorescence emission spectroscopy. The recombinant protein binds to phospholipid vesicles (PS, PA, PG and PE, but not to PC) in a calcium-dependent manner, but requires significantly higher calcium concentrations than other annexins. This process involves a structural rearrangement of the protein with exposure of the Trp residue similar to that detected at high calcium concentrations in the absence of PS. In vivo, this annexin is myristoylated in the N-terminal Gly and may interact with other proteins, probably modifying the calcium requirements for the interaction with membranes.

## 6-31

### Influence of n-terminal modifications of annexin a13 on its structural-functional properties

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Annexin-A13 is one of 12 vertebrate subfamilies in the annexin superfamily of calcium/phospholipid binding proteins. Structurally, annexins present a homologous protein core with four (or eight) internal repeats and a highly variable N-terminal extension responsible for the regulation and the specific functions of individual annexins. Annexin-A13 can be expressed as two splice variants that differ in an insertion of 41 amino acids (A13b) in position 6, not present in the short variant (A13a). The N-terminal Gly is myristoylated in vivo in both isoforms and they appear associated to raft microdomains. This annexin is mainly expressed in differentiated intestinal epithelial cells, being annexin-A13b involved in the apical transport of vesicles from the trans-Golgi network and A13a in both apical and basolateral transport. In order to analyze the influence of N-terminal modifications of annexin-A13 in its properties, we have expressed both splice variants in E. coli and coexpressed annexin A13b with N-myristoyl transferase, allowing the production of the recombinant myristoylated form. A comparative circular dichroism and fluoresecence emission spectroscopic analysis of both isoforms reveals that there are no significant differences in their secondary structure; calcium binding induces similar structural rearrangements, although the A13a variant shows higher thermal stability both in the absence and presence of calcium (6-7C). Calcium requirement for binding to PS vesicles is also lower for the short isoform (50 vs 300 microM). On the other hand, the myristoylated A13b isoform seems to bind to different phospholipid vesicles (PS, PA, PG, PE, PC) in a calcium-independent manner including raft-like vesicles.

## 6-32

### How Ca Affects Dynamics and Biochemical Functioning of Horseradish Peroxidase

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Native HRPC binds two Ca2+ions distal and proximal to the heme group. The two Ca-binding sites were studied by experimental and computational methods. Long time molecular dynamics simulations (MDS) unravel that the distal site is embedded in a more rigid region of the enzyme, whereas the proximal site is close to the protein surface in a more flexible region. Long time MDS were performed on the solvated and fully energy minimized structures of the protein in the native state and also without one or two endogenous calcium ions. The structure of heme pocket of these three different protein structures was significantly different. The analysis of the deviation of the heme group from planarity showed the consequence of these changes. It was shown that the heme distortion is the strongest in the native state. Results by optical spectroscopy at 10K support the predictions based on the model studies. The stabilization effect of Ca2+ was also studied by pressure denaturation experiments and by isothermal titration calorimetry measurements to compare the substrate binding affinity of the native and Ca2+-depleted forms of the enzyme.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-33

### Measurement of the isothermal compressibility of hydrated myoglobin by small-angle neutron scattering

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Small-angle neutron scattering experiments were carried out at room temperature on pD 6.6 solutions of horse heart azidometmyoglobin (MbN3)in order to determine the second virial coefficient of the protein solution and the actual radius of gyration of the protein. The results shows that the interactions between the macromolecules are always strongly repulsive, even if their magnitude decreases with increasing pressure, whereas the radius of gyration of the protein remains constant. This indicates that the compactness of MbN3 is not altered by pressures up to 300 MPa. However it is possible that a molten globule forms at the highest pressures. Taking advantage of the pressure-induced contrast variation of the protein these experiments allow the partial specific volume of MbN3 to be determined as a function of pressure. Its value decreases by about 5.4% between atmospheric pressure and 300 MPa. In this range the isothermal compressibility of hydrated MbN3 is found to be(1.6+/-0.1) 10^-4 MPa^-1. This result will be compared with those obtained by means of other techniques. Reference: C. Loupiac, M. Bonetti, S. Pin & P. Calmettes, Eur. Jour. Biochem. 269 (2002) 4731-4737.

## 6-34

### Structure-function correlation of a membrane protein in a 'jig saw' approach: Vpu from HIV-1

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Structural information of a protein in general is a key step forward in elucidating the mechanism of function. Depending on the method applied, investigations on the whole protein can be demanding. We are therefore using only parts of the viral membrane protein Vpu from HIV-1 to gain an insight into the mechanisms of function of the full protein (a 'jig saw' approach). Vpu is a membrane protein of 81 amino acids encoded by HIV-1. Vpu function is to amplify the release of progeny virions via two distinct pathways: (i) docking of Vpu to CD4 with its cytoplasmic part in the ER and (ii) ion channel activity via self-assembly near the plasmamembrane. The structural motif of Vpu is: a helical transmembrane (TM) domain followed by a second helix (helix-2) parallel to the membrane surface, a third helix most likely in lose contact with the membrane and a short motif at the C terminal end either a turn or helix. Conductance measurements verify our computational data on the Vpu TM domain. This forms the basis for further investigation using MD simulations to predict structural stability of mutants and their potential for conducting ions (manuscript in preparation). In a next step we extend our simulations on a Vpu construct which includes the TM helix and a second helix (helix-2). Our simulations are indicative that helix-2 may act as a 'peptide float' riding on the lipid bilayer. Further simulations with helix-2 only support this idea (manuscript in preparation).

## 6-35

### Probing the binding site of amiloride derivatives on vpu bundles from HIV-1

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Vpu is a membrane spanning auxiliary protein of 81 amino acids encoded by HIV-1. Its role is to enhances particle release from the infected cell. This is achieved via two mechanisms: (i) docking to CD4 with its cytoplasmic domain initiating CD4 down regulation and (ii) exhibition of ion channel activity which is connected with its capability to form homo-oligomers and linked to the helical transmembrane (TM) domain. Probing the binding site of inhibitors to proteins is essential for future drug development. Here we present a study on binding of a putative channel blocker to bundles consisting of an assembly of helices corresponding to the TM domain of Vpu, forming a putative pore. In addition docking studies on single helices are also conducted. The AutoDock program was used to find the binding sites of the drugs and their conformation upon docking. Docking simulations are performed for six different Vpu proteins with amiloride and the derivative cyclohexamethylene amiloride (HMA), with the latter being a channel blocker (manuscript in preparation). The dependence of the protonation state of the drugs on binding and their conformation are also investigated. The results show that the binding site is proximal to the Ser residue towards the C-terminal end of the TM helix. The results suggest that the difference in binding conformations of amiloride and HMA in the Vpu bundle may explain the experimentally found inhibition of channel activity by HMA but not by amiloride.

## 6-36

### Design, expression and characterisation of a chimeric form of the alpha-spectrin sh3 domain including a peptidic ligand.

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SH3 domains are small protein modules that interact with proline-rich sequences with moderate affinity. Several high-affinity, peptidic ligands for the abl-SH3 domain have been designed recently. One of these peptides named p41, with sequence APSYSPPPPP, also binds with moderate affinity to the alpha-spectrin SH3 domain, for which no ligands have been known to date. We have used protein-engineering methods to design a chimeric SH3 domain, which includes in its sequence the p41 chain in such a way that it docks into the binding pocket. To do so we started with a circular permutant of the alpha-spectrin SH3 domain (cpSH3), previously designed by us, and extended its sequence from the C-terminus with 13 additional residues, consisting of a 3-residue linker plus the p41 sequence. We refer to this chimera as cpSH3-p41. The aim of this design was to obtain a single-chain small protein that could serve as a tool for the optimization of interactions between the SH3 domain and the ligand sequence by a combination of site-directed mutagenesis and thermal stability studies. We have made a preliminary characterization of the structural and stability properties of cpSH3-p41 and have compared it with cpSH3 using differential scanning calorimetry (DSC), circular dichroism (CD) and homonuclear NMR. cpSH3-p41 is more stable than cpSH3 by 7-10 C and the structural data indicate that the p41 sequence adopts a well-ordered poly-proline II helix in cpSH3-p41, similarly to what it is usually found in the structures of the complexes formed between proline-rich ligands and SH3 domains.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-37

### Molecular dynamics simulation meets experiment: EPR spectra simulation of spin labeled proteins

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EPR-spectroscopy in conjunction with site directed spin labeling [1] is a highly appropriate method to analyse the structure, dynamics or conformational changes of proteins. Using site directed mutagenesis technique EPR-sensitive spin labels are specifically linked to desired sites in the protein. In the first place the spectra depend on the motional freedom of the spin label giving information about the local structure of the protein under physiological conditions. The preparation of a set of protein mutants with different spin label positions leads to the identification of secondary and tertiary structure elements. EPR spectra calculations based on molecular dynamics simulations facilitate to verify or refine a predicted computer aided model of the local protein conformation by comparing the simulated and measured EPR spectra. The EPR spectra simulation algorithm is presented using data received from molecular and stochastic dynamics simulations [2,3]. Like in the sample preparation a spin label is modeled at the corresponding site in the computerized protein structure. Potentials which determine the spin label motion are obtained from the molecular dynamics trajectory and provide the basis for the spectra simulation. A model [4] of the EF-loop structure of the membrane protein bacterio-rhodopsin was validated using this EPR spectra simulation method. It has been shown that this algorithm can also be used to analyze conformational changes [3]. References [1] Hubbell, W. L., Mchaourab, H. S., Altenbach, C. and Lietzow, M. A. (1996). Structure 4, 779-783 [2] Steinhoff, H.-J. and Hubbell, W. L. (1996). Biophysical Journal 71, 2201-2212. [3] Steinhoff, H.-J., Mueller, M., Beier, C. and Pfeiffer, M. (2000). J. Mol. Liquids 84, 17-27 [4] Essen, L.-O. and Oesterhelt, D. (1998). Proc. Natl. Acad. Sci. USA 95, 11673-11678.

## 6-38

### Kinetics of different processes involved in the formation of insulin amyloid fibrils

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A wide range of pathologies, including systemic amyloidoses, type II diabetes, and neurodegenerative diseases, is characterized by the anomalous self-assembly and deposition of proteic material into insoluble (ordered) supramolecular structures, such as fibrils or aggregates of fibrils. Fibrillation is a nucleation-dependent polymerization process strongly affected by pH, ionic strength, temperature, monomer concentration and the presence of metal ions. In the present work, we focused on a well-known protein, insulin, as a model to study the kinetics of amyloid aggregation. Static and dynamic light scattering experiments were performed at acid pH, and in a wide range of temperatures and concentrations to follow the formation of different structures, starting from monomeric proteins in solution and up to elongated fibrils on the scale of hundreds of nanometers. Optical microscopy and small angle light scattering were also used to study the structures of even more larger aggregates (on the scale of microns). The final structure of aggregates, as well as their growth kinetics, was found to depend upon the initial thermodynamic conditions (such as temperature and concentrations). Time-resolved experiments allowed to identify the different processes involved (nucleation, elongation, flocculation) and to relate their interplay to aggregation kinetics and final structure of aggregates.

## 6-39

### Role of the lisine rich cluster of the c2 domain of pkc alpha

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Protein kinase C (PKC) comprises a large family of serine/threonine kinases, which is activated by many extracellular signals and plays a critical role in many signal-transducing pathways in the cell. Recent structural studies have suggested that the highly positive charged beta3-beta4 sheets of the C2 domain of PKCalpha can interact electrostatically with negatively charged phospholipids located at the membrane surface. In this study, we focus on the characterization of the interaction mechanism between this lysine rich cluster and different negatively charged phospholipids, including phosphatidylserine and phosphoinositides and the consequent enzyme activation. Site-directed mutagenesis on this site has revealed that the domain can play two different roles depending on the phospholipid present in the membrane vesicles. Thus, this area of the domain establishes an intramolecular interaction with an aspartic residue located in the C1 domain maintaining the enzyme in a partially inactive conformation. Alternatively, the enzyme can be activated specifically by PtdIns(4,5)P2 through this site independently of phosphatidylserine.

## 6-40

### Ultrafast changes in the protein absorption band of bR

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Electric fields and dielectric responses in proteins govern their biological functions. The aminoacid Tryptophan (Trp), an excellent probe for electric fields, is naturally present in some proteins. Two Trps sandwich the retinal chromophore in the proton pump bacteriorhodopsin (bR) and the retinal's dipole moment can be instantaneously increased by light absorption. The response of these Trps due to excitation and relaxation of the dipole moment of retinal was the subject of our fs-time resolved studies. Our pump-probe laser set-up consisted of two noncollinear optical parametrical amplifiers. One was centered at 560 nm for excitation of the retinal, the second was frequency doubled to yield the UV-probe pulses (260 nm to 310 nm). The cross-correlation between the two pulses was 90 fs. Wild-type bR was studied in purple membrane suspensions circulated by a flowcell through a peristaltic pump. Thus, the sample was refreshed between subsequent laser shots. The single-shot detection set-up detected small absorption changes in the UV-region. For wavelengths > 300 nm, we observe an additional absorption, consistent with an internal Stark effect of Trp86. After an instantaneous rise, the signal shows a 500 fs decay, the latter corresponding to the decay of excited retinal. For wavelengths < 295 nm, separating the contribution of Trp bleach and higher excited state dynamics of retinal is not straight forward. Therefore additional measurements on native bacteriorhodopsin and also on Trp-mutants are being carried out.

## *Posters*

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## 6-41

**Vpu from HIV-1 interaction with an amiloride derivative: a docking and molecular dynamics study**

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Vpu is an 81-residues membrane-protein with one transmembrane (TM) segment that is encoded in HIV-1. It associates with the Golgi apparatus and endoplasmic reticulum and has two major biological functions: (i) selective degradation of CD4 through the C-terminal cytoplasmic part and (ii) enhancement of viron release via formation of an ion channel. This work focuses on the ion-channel activity of Vpu and how it can be inhibited. Conductance measurements have shown that amiloride derivatives can inhibit the ion channel activity of Vpu. To investigate the inhibition mechanism docking of cyclohexamethylene amiloride was performed on a Vpu pentamer of the first TM domain combined with Molecular Dynamic (MD) simulations to add conformational flexibility to the protein, and study the dynamic behaviour of the bound drug. A 12ns simulations was performed on the ion-channel model inserted in a hydrated lipid bilayer (manuscript in preparation). During the MD, the drug remains bound near the docking site, oriented along the channel with the hydrophobic part of the molecule pointing inside the pore. MD simulations on the same system was performed, removing the partial charge on the drug. The drug remains bound to the protein, although its conformation and orientation changes. This suggests that electrostatic interactions are not the major component for the interaction of the drug with Vpu. Several simulations with the drug at arbitrary sites are done as a control. When placed far away from the binding site, the drug tends to go out of the channel, remaining at the membrane/solvent interface.

## 6-42

**Interaction of dystrophin rod domain and lipid membranes : affinity, electrostatic nature and involvement of tryptophan residues.**

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Dystrophin in the protein which is lacking in Duchenne muscular dystrophy. Its structure and function are not precisely known. We undertaken a study of the interaction of the second repeat of the central rod domain of dystrophin with lipids. To assess dystrophin rod domain membrane interactions, tryptophan residues properties of two recombinant proteins of the rod domain were examined by proton NMR and fluorescence methods in presence of membrane lipids. The first protein is a partly folded protein as inferred from NMR, tryptophan fluorescence emission intensity and the excited state lifetime. By contrast, the second longest protein is a folded compact protein. Tryptophan fluorescence quenching showed that both proteins are characterised by structural fluctuations with their tryptophan residues only slightly buried from the surface. The tryptophan fluorescence properties of the longest protein changed dramatically in presence of anionic phospholipids demonstrating a specific interaction between this protein and membrane lipids while only minor changes were observed for the non folded protein. By ultrafiltration methods, the affinity constant of the longest protein is showed to be high. The nature of the interaction is at least partially electrostatic as indicated by the dependence of the affinity upon NaCl and pH. We propose a new scheme of dystrophin molecule interactions including the interaction with plasma membrane.

## 6-43

**Ca+2-induced contraction of 3 nm diameter biofilaments in vorticellidae spasmoneme and their chemical modification**

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Peritrich ciliates possess a unique contractile system of 3 nm diameter filaments in the spasmoneme. In the present study, we have identified histidine and tyrosine residue(s) critical for spasmoneme contraction by using glycerinated stalk of Vorticella. We observed concentration-dependent inhibition of spasmoneme contraction in the presence of reversible histidine-modifying reagent, namely diethyl-pyrocarbonate (DEPC). Further more, contractility degree of modified spasmoneme could be partially restored by hydroxylamine treatment. The absorption of modified spasmoneme protein(s) at 244 nm increased with the rising of DEPC concentration and decreased after with the adding of hydroxylamine. In the chemical modification of tyrosine residues in Vorticellidae spasmoneme with tetranitromethane (TNM), we found here that TNM treatment at its concentration range of 20-80uM caused an irreversible and concentration-dependent decrease in the stalk contractility at 0 degree and pH 8.0. Ca2+ adding before modification reagent could prevent the spasmoneme contraction from inhibition of DEPC and TNM modification. Those results suggested that histidine and tyrosine was necessary for spasmoneme contraction. The Ca2+ binding ability of spasmin was not inhibited by DEPC modification, which implied that there is another protein essential for spasmoneme contraction.

## 6-44

**Biophysical investigation of intermolecular interactions of the human centrin 2**

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Human centrin 2 (HsCen2) is a Ca2+-binding protein of the EF-hand family, usually associated with the centrosomes, that is required for the normal centriole duplication during the mitosis. Recent biochemical evidence suggests that a fraction of HsCen2 localized in the nucleus may interact with the XPC protein, a subunit of the Nuclear Excision Repair complex. We identified two putative binding sites in the XPC sequence and demonstrated (using Isothermal Titration Calorimetry) that one of the corresponding peptides (P1-XPC: N847-R863) binds with high affinity to both HsCen2 and to CaM, in a Ca2+-dependent manner. This peptide also binds, with a similar affinity, to a C-terminal construct of HsCen2 (SC-HsCen2: T94-Y172), indicating that the peptide mainly interacts with the C-terminal half. NMR, fluorescence and Circular Dichroism were used for the structural and dynamic characterization of the interaction of P1-XPC with SC-HsCen2 domain in the presence of Ca2+. CD experiments show that P1-XPC is highly disordered in solution but the N-terminal moiety forms an a-helical structure (confirmed by NMR experiments) upon binding to SC-HsCen2. As shown by fluorescence of the peptide Trp side chain, the indole ring is deeply embedded in the hydrophobic cavity of the domain. Heteronuclear, multidimensional NMR experiments enabled us to determine the three-dimensional structure of the SC-HsCen2/P1-XPC complex. Light scattering, NMR, microcalorimetry and electron microscopy experiments revealed that HsCen2 polymerizes under Ca2+ and temperature control. Interactions with P1-XPC perturbs considerably the aggregation process, suggesting that an equilibrium between intermolecular interactions and homo-polymer formation may have a physiological significance.

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## 6-45

### Beta-lactoglobulin stability and hydration in the presence of trehalose by time resolved fluorescence

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Time resolved fluorescence of the probe acrylodan, covalently bound to the unique free cysteine of beta-lactoglobulin (BLG) has been applied in order to ascertain the stabilizing role of the disaccharide trehalose on BLG against its chemical denaturation, both at native and acidic pH. The changes of acrylodan fluorescence lifetime versus guanidinium chloride concentration reveal BLG sigmoidal denaturation profiles which depend upon the trehalose concentration. When adding trehalose to the solution in the presence of denaturants the protein transition midpoint shifts toward higher denaturant concentration. This effect can be described by a two state model whose parameters indicate that a nearly 60% increase in the denaturation free energy is induced, independent of trehalose concentrations and pH values. Fluorescence anisotropy measurements performed on BLG solutions, reveal that the internal dynamics is largely affected both by the sugar, which makes the acrylodan environment more rigid, and by the denaturant that acts in the opposite way. Measurements of the overall rotational diffusion of BLG suggest that the hydrodynamic properties of the solution in the proximity of the protein are significantly altered by trehalose and tentative mechanisms are discussed.

## 6-46

### Transient state protein kinetics by time-resolved macromolecular x-ray crystallography

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Proteins are the catalysts of life. To understand their function the meta-stable structures of the intermediates which rise and decay during the reaction must be determined. With time-resolved macromolecular crystallography the reaction can be followed without perturbation at ambient temperature. Both, structure and kinetics can be determined simultaneously. After the initiation of a reaction in the protein crystal, Laue data sets are collected at time points equally spaced on a logarithmic time scale. However, it is not obvious how to extract structures and kinetics from the data. At any time point, several intermediates mix into each other and prevent the interpretation of the maps. The time information can be used to separate the admixture. A component analysis, the singular value decomposition (SVD), was assimilated to work with time-dependent difference maps. Numerous simulations with realistic mock data were performed to show the feasibility of the SVD-driven analysis. (i) The time-information can be used as noise filter. (ii) The pure, time-independent difference electron densities of the intermediates can be extracted. From these, the structures can be determined. (iii) The determination of a suitable chemical, kinetic mechanism is initiated, which can be completed after the structures of the intermediates have been determined. The SVD-driven analysis was applied to realistic data consisting of 15 time-dependent difference maps from time-resolved Laue experiments on Photoactive Yellow Protein on a time scale from 5 μs to 100 ms. 3 intermediate states were structurally characterized and a confined set of plausible chemical, kinetic mechanisms was determined.

## 6-47

### Tracing structural intermediates in ligand binding

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Specific protein-ligand interactions are central to biological control. Although structure determination provides important insight into these interactions, it does not address dynamic events that occur during binding. While many biophysical techniques can provide a global view of these dynamics, NMR spectroscopy yields information about synergistic rearrangements and interactions that determine specificity at the molecular level. We have analyzed line shapes of NMR signals to detect long-lived intermediates that occur upon ligand binding. The nature of these intermediates is substantially different for different ligands and mutants of a SH2 domain. Specificity and binding affinity depends on the stability and life time of intermediates. This analysis provides a novel view of the kinetic course of protein-ligand interactions and may change perspectives for drug design if inhibitors can be designed to block intermediate states or sites which trigger conformational rearrangements required for binding. References T. Mittag, B. Schaffhausen and U. Günther. Tracing Structural Intermediates in Ligand Binding. Submitted U. Günther, B. Schaffhausen and T. Mittag, Probing SH2 Domain Ligand Interactions by differential line broadening. Biochemistry 41, 11658-11669 (2002) U. Günther, B. Schaffhausen, NMRKIN: Simulating line shapes from two-dimensional spectra of proteins upon ligand binding. J. Biomol. NMR, 22, 201-209 (2002)

## 6-48

### Role of the core domain in the activation of nucleoplasmin, a nuclear chaperone.

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Nucleoplasmin (NP) mediates nucleosome assembly by removing basic proteins from sperm chromatin and exchanging them for histones. This chaperone activity is modulated by phosphorylation of NP at multiple sites. NP is pentameric, each monomer consisting of two domains: a core and a tail. The core domain adopts a beta-sandwich structure and forms a stable ring-like pentamer. The tail domain contains a motif rich in negatively charged residues (the "polyglu"), putatively involved in binding of NP to basic proteins, and a nuclear localization signal. Despite lacking the polyglu region, the isolated core domain of the hyperphosphorylated protein is enough to bind basic proteins and decondense chromatin. In this study, we have generated active recombinant core domains by means of mutation of possible phosphorylation sites for aspartic acid, that is, mimicking the effect of phosphorylation. By contrast to the wild type core, the mutants are active. We have characterized the conformation and stability of these mutants: a correlation between activity level and destabilization of the protein structure is observed. The mutated residues locate in flexible or loop regions exposed on the "distal face" of the core pentamer, where an acidic region shorter than polyglu is also found, indicating that phosphorylation might activate NP generating a strong localized negative potential. Our results indicate which residues could be key phosphorylation targets and suggest that the core domain of phosphorylated NP contributes together with the polyglu containing tail in displaying a binding surface for basic proteins on the NP pentamer.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-49

### Simulations of Outer Membrane Proteins: Comparing X-Ray and NMR Structures

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The structures of membrane proteins can be determined by X-ray crystallography or, more recently, by NMR of mixed protein/detergent micelles. Both of these techniques yield static average structures in a non-membrane environment. Molecular dynamics (MD) simulations may be used to study the conformational dynamics of membrane proteins in a lipid bilayer environment. We are using MD simulations to compare the conformational dynamics of two small bacterial outer membrane proteins, OmpX and PagP, both of which have a characteristic beta-barrel membrane spanning domain. OmpX is a relatively rigid outer membrane protein, whose function is implicated in virulence, mediating the adhesion of bacteria to host cells and thus neutralising host defence mechanisms. Conversely, PagP is suggested to be a more flexible outer membrane protein with large extracellular loops that contain a catalytic triad, whose function is to transfer a palmitate chain to lipid A. This in turn confers resistance to cationic antimicrobial peptides produced during the host immune response. By carrying out multi-nanosecond simulations of these proteins embedded in lipid membrane, using starting structures from both NMR and X-ray experiments, quantitative analysis of the protein dynamics in the simulations can be compared in order to provide a deeper understanding of the quality of simulations resulting from different starting structures.

## 6-50

### Purification and properties of TrwB, a hexameric, ATP-binding integral membrane protein essential for R388 plasmid conjugation

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TrwB is an integral membrane protein linking the relaxosome to the DNA transport apparatus in plasmid R388 conjugation. Native TrwB has been purified in monomeric and hexameric forms, in the presence of dodecylmaltoside from overexpressing bacterial cells. A truncated protein (TrwBDN70) that lacked the transmembrane domain could be purified only in the monomeric form. Electron microscopy images revealed the hexameric structure and were in fact superimposable to the previously published atomic structure for TrwBDN70. In addition, the electron micrographs showed an appendix, 25 A wide, corresponding to the transmembrane region of TrwB. TrwB was located in the bacterial inner membrane in agreement with its proposed coupling role. Purified TrwB hexamers and monomers bound tightly the fluorescent ATP analogue TNP-ATP. A mutant in the Walker A motif, TrwB-K136T, was equally purified and found to bind TNP-ATP with a similar affinity to that of the wild type. However, the TNP-ATP affinity of TrwBDN70 was significantly reduced in comparison with the TrwB hexamers. Competition experiments in which ATP was used to displace TNP-ATP gave an estimate of ATP binding by TrwB (KdATP = 0.48 mM for hexamers). The transmembrane domain appears to be involved in TrwB protein hexamerization and also influences its nucleotide-binding properties.

## 6-51

### Three pharmacophores for the multispecific recognition of molecules by the multidrug transporter P-glycoprotein

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Pglycoprotein (Pgp) is an ABC transporter expressed in plasma membrane and responsible for the active efflux out of the cell of a large number of amphiphilic molecules of unrelated chemical structures. Pgp is involved in cell and tissue detoxification process against various xenobiotics and pharmaceuticals, with a key role in the multidrug resistance (MDR) of some tumor cells against drugs used in anticancer chemotherapy. We investigated the molecular properties involved in the binding selectivity of Pgp by a molecular modeling approach using various substrates combined with an enzymological study of the Pgp ATPase activity modulations induced by these substrates. Modeling of intramolecular distribution of the hydrophobic and polar elements of the tested molecules made it possible to superimpose some of these elements. These molecular alignments were correlated with the observed mutual exclusions for binding on Pgp, leading to the characterization of three different pharmacophores, defined by their respective consensus recognition elements. On each of these pharmacophores the ligands compete with each other. The typical MDR associated molecules, verapamil, cyclosporin A and actinomycin D, bind to pharmacophore 1, whereas vinblastine bind to pharmacophore 2. In addition, some polycyclic and planar molecules, such as daunorubicine, allowed to define a third pharmacophore. Thus, the multispecific binding pocket of Pgp is considered as composed of sites which bind ligands according to the distribution of their hydrophobic and polar elements rather than their precise chemical motifs. The existence of three pharmacophores increases the possibilities for recognition of a broad diversity of chemical structures.

## 6-52

### The catalytic proficiency of orotidine 5'-monophosphate decarboxylase: an hybrid QM/MM study

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The decarboxylation of orotidine 5'-monophosphate (OMP) to uridine 5'-monophosphate by orotidine 5'-monophosphate decarboxylase (ODCase) is an essential step in the nucleic acids biosyntesis. We have revised the ODCase catalytic mechanism by using highly accurate classical molecular dynamics (MD) and state-of-the-art hybrid quantum/classical molecular dynamics MD (QM/MM) simulations. Classical simulations have been performed on the nanoseconds time scale by using the Amber force field and the Ewald summation for the electrostatics. QM/MM simulations have been performed combining the Density functional theory-based Car-Parrinello method with classical MD simulations following the approach recently proposed by Rothlisberger and coworkers [J. Chem. Phys. 116, 6941 (2002); J. Phys. Chem. B 106, 7300 (2002)]. Decarboxylation have been investigated using the multiple-steering MD method [Jarzynski Phys. Rev. Lett. 78, 2690 (1997)]. We have found that the mechanisms based on direct protonation of OMP as initial step are unlikely because of the absence of sufficiently acidic groups nearby the OMP substrate and therefore, should be ruled out. Instead, from our calculation is evident that the most likely mechanism involves a direct decarboxylation. However, the catalytic mechanism is not driven by ground-state destabilization by electrostatic stress [PNAS 97, 2017 (2000)]. Indeed, it is found that the OMP-Lys-Asp-Lys-Asp charged array is very stable. We proposed that the rate enhancement of ODCase is due to transition state stabilization [Warshel et al., Biochemistry 39, 14728 (2000)], and it is assisted by the charged network, which drives the rupture of the C-C bond.

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## 6-53

### Nucleoplasmin, a nuclear chaperone whose stability depends on its phosphorylation level. An equilibrium unfolding study

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The stability of recombinant and natural nucleoplasmins (NP) against common protein denaturants has been studied, following the effect of urea, guanidinium chloride (GdnHCl) and guanidinium thiocyanate (GdnSCN) on the fluorescence properties of those variants. Chemical denaturation of the proteins induces a large red shift (~ 15 nm) and a decrease of their fluorescence intensity. The effect of the denaturant ranked urea < GdnHCl < GdnSCN, the latter being the most effective for protein unfolding. Unfolding equilibrium curves were obtained by plotting the maximum emission wavelength as a function of denaturant concentration. The same type of curve was observed when steady-state anisotropy values of the protein were plotted. The experimental points collected from each curve could be fitted by a two-state model. There was a good correlation between the Cm values obtained from the analysis of both parameters. Complete unfolding of a protein is tipically characterized by the loss of secondary structure. We monitored by FTIR the secondary structure of recombinant NP (rNP) in the presence of denaturing concentrations of the three denaturants. When unfolding experiments at equilibrium were performed at different rNP concentrations, the midpoints of the transitions were almost unchanged. This result is consistent with a sequential unfolding mechanism, characterized by three states with either the monomerization step (5N) preceding the denaturation step (5U) or the unfolding of the pentamer (U5) taking place before the denaturation process (5U). We are further characterizing the putative NP unfolding mechanism by cross-linking and analytical centrifugation studies

## 6-54

### Leukemia Inhibitory Factor, cardiotrophin-1 and oncostatin M share a common binding site in the Ig-like domain of LIF receptor

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The leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and oncostatin M (OSM) are four helix bundle cytokines acting through a common heterodimeric receptor composed of gp130 and LIF receptor (LIFR). Binding to LIFR occurs through a binding site characterized by a FxxK motif located at the N-terminus of helix D (site III). The Ig-like domain of LIFR was modeled and the physico-chemical properties of its Connolly surface were analyzed. This analysis revealed an area displaying properties complementary to those of the LIF site III. Two residues of the Ig-like domain of LIFR formed a mirror image of the FxxK motif. Molecular docking of LIF to the Ig-like domain of LIFR using HEX (Ritchie and Kemp, 2000) corroborated aromatic-aromatic and salt bridge interactions. Engineered LIFR mutants in which either or both of these two residues were mutated to alanine were transfected in BA/F3 cells containing gp130. The double mutation was required to totally impair (LIF and CT-1) or partially impair (OSM) the proliferative response induced by the cytokines. These results were corroborated by Scatchard analysis of LIF, CT-1 and OSM binding to BA/F3 cells. Our results indicate that LIF, CT-1 and OSM share an overlapping binding site located in the Ig-like domain of LIFR. The different behavior of LIF and CT-1, on one side, and of OSM, on the other side, can be related to the different affinity of their site III for LIFR.

## 6-55

### Dynamic transition associated with the thermal denaturation of a small beta protein

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We studied the temperature dependence of the picosecond internal dynamics of an all beta protein, neocarzinostatin, by incoherent quasielastic neutron scattering (IQENS). IQENS directly probes the internal dynamics of biomolecules on the picosecond time scale, providing information on diffusive motions and the geometry of the motions observed. Measurements were made between 20 C and 71 C in heavy water solution. At 20 C only 33 per cent of the non-exchanged hydrogen atoms show detectable dynamics, a number very close to the fraction of protons involved in the side chains of random coil structures, therefore suggesting a rigid structure in which the only detectable diffusive movements are those involving the side chains of random coil structures. At 61.8 C, while the protein structure is still native, slight dynamic changes are detected which could reflect enhanced backbone and beta sheet side chain motions at this higher temperature. Conversely, all internal dynamics parameters (amplitude of diffusive motions, fraction of immobile scatterers, mean-squared vibration amplitude) rapidly change during heat-induced unfolding, indicating a major loss of rigidity of the beta sandwich structure. The number of protons with diffusive motion increases markedly, whereas the volume occupied by the diffusive motion of protons is reduced. At the half-transition temperature (T=71 C) most of backbone and beta sheet side chain hydrogen atoms are involved in picosecond dynamics.

## 6-56

### Structural and conformational changes involved in thermal aggregation processes of bovine and human serum albumins

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To characterize the mechanisms and pathways of proteins aggregation, we have investigated the structural and conformational changes involved in thermal aggregation of two essentially alpha helical proteins, BSA and HSA. The experimental approach is based on the combined use of FTIR spectroscopy and dynamic light scattering. By using FTIR spectroscopy and deuterated samples, we are able to appreciate, in both proteins, the kinetics of the exchange between Amide II and Amide II' as first step that drive towards the aggregation. These results suggest an initial partial unfolding at tertiary structure level involving not exchangeable hydrogens confined in the inner part of proteins. We are also able to follow the changes of the Amide I' band profile as a function of time. In particular, we observe a decrease of the spectral component at 1650 cm-1, attributed to alpha helix structures, and the simultaneous growth of two bands at 1616 and 1680 cm-1, attributed to aggregated beta structures. All these spectral features are similar for both proteins and depend on temperature and pH values. These structural changes are put in relation with the extent of aggregation by using dynamic light scattering measurements, to follow the growth, as a function of time, of hydrodynamic radius of the aggregates. The reported data show that at higher pH values the aggregation proceeds through oligomers formation essentially driven by beta-structures aggregation, while, at lower pH values, other rapid mechanisms of aggregation occur, something like sticking between molecules.

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## 6-57

### Thermal aggregation processes seen by intrinsic and extrinsic probes in different globular proteins

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Globular proteins, when heated, partially unfold and through conformational changes at tertiary and secondary structure level tend to aggregate. In order to study how these conformational changes depend from the native protein structure we report here a study of aggregation processes of Bovine Serum Albumin (BSA), Human Serum Albumin (HSA) and beta-Lactoglobulin (BLG) in low concentration regime (<10uM) and at temperatures between 60 and 80 C. BSA and HSA present homologous structures, essentially alpha helix while BLG presents about 70% of beta structures. The intrinsic fluorescence of these proteins is mainly due to tryptophans whose quantum yield is highly dependent on the particular environment. HSA owns only one tryptophan (trp214) buried in a internal part of domain II. BSA, instead, owns two tryptophans: the first (trp214) localized in a microenvironment similar to HSA (domain II) , and the second (trp134) localized in a hydrophobic pocket near the surface of the molecule. Finally, BLG owns two tryptophans localized in environments completely different by the foregoing proteins. The first (trp61) is part of an external loop and the second (trp19) is at the bottom of the central hydrophobic calyx. Emission kinetics of these intrinsic probes in the three proteins show different behaviours related to the different environments in their surrounding and are discussed in terms of tertiary and secondary structural changes. These last are also followed by using CD measurements. We also study emission kinetics of ANS, whose variations can be related to changes involving hydrophobic regions of the proteins.

## 6-58

### Different steps of thermal aggregation of lysozyme

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Lysozyme (Lys) is a small enzymatic protein that, in particular conditions, forms macromolecular aggregates. In general, aggregation processes compete with the correct folding pathway and commonly proceed in almost two different steps, reversible and irreversible, leading to the formation of aggregates like oligomers or fibrils. To characterize the different mechanisms and pathways of this process we report an experimental study on the thermal aggregation of Lys (60C-80C), by using different spectroscopic techniques,. To take care of tertiary structure conformational changes, we study the tryptophanil emission and the fluorescence properties of ANS dye, whose quantum yield strictly depends on the hydrophobicity of its environment. In particular, we can distinguish irreversible and reversible processes following emission spectra of ANS. To study conformational changes at secondary structure level, we use FTIR measurements. In particular, we observe the increase of aggregated beta structures bands (1616 and 1680 cm-1) only at temperatures higher than 70 C, while, at lower temperature, aggregation pathway proceeds without this kind of structural variations. Moreover, complementary information about secondary structure is obtained by CD measurements. At lower concentrations (<10 uM), as monitored by fluorescence measurements, aggregation leads only to oligomer formation, while, at higher ones (about 4mM) aggregation also leads to gel formation, as measured by FTIR spectroscopy. In both concentration regime we observe that Lys presents an high stability of the secondary structure and that aggregation process can begin also only through conformational changes of tertiary structure.

## 6-59

### Solid-state 205Tl NMR studies of interactions between Tl+ and Na+,K+-ATPase membranes

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The Na+,K+-ATPase is a membrane-bound enzyme transporting 3 Na+ out of and 2 K+ into the cell against their chemical gradients at the expense of hydrolysis of one ATP molecule. The K+-occluding E2 conformation is spontaneously formed in the presence of K+ or one of its congeners, i.e. Rb+, Cs+, or Tl+, which are NMR sensitive nuclei. In this study the Tl+ binding to Na+,K+-ATPase membranes from shark rectal gland and pig kidney has been examined using 205Tl magic-angle-spinning (MAS) solid-state NMR. The spectra are interpreted by means of Tl+ binding isotherms calculated from occlusion and equilibrium experiments with radioactive 204Tl+. At a low Tl+/enzyme ratio the NMR signal is dominated by the specific Tl+ binding to occlusion sites. At a higher Tl+ concentration a non-specific component increases leading to a resonance, which consists of both specific and non-specific Tl+ binding. Addition of excess of K+ displaces much of the occluded Tl+ resulting in an NMR signal dominated by non-specific Tl+ binding. This K+ addition moves the NMR signal towards higher chemical shift. It is thus possible to distinguish between NMR spectra containing mainly specifically bound and non-specifically bound Tl+. All the 205Tl NMR spectra consist of narrow resonances. This absence of anisotropic line shape indicates mobility of Tl+ in the occlusion sites fitting well with the flickering gate model introduced by Forbush [J. Biol. Chem., 262, 11116-11127, 1987].

## 6-60

### The interaction of nucleoplasmin with basic proteins of the sperm nuclei

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Nucleoplasmin (NP) is a thermostable and pentameric protein belongs to a family of acidic proteins and was the first identified molecular chaperone due to its ability to mediate nucleosome assembly . Each 200 aa identical monomer folds into two domains: 80 residues protease sensitive C-terminal domain (the tail), that contains the nuclear location signal (NLS) and the polyglutamic tract, and a protease resistant N-terminal domain (the core). The core domain is able to oligomerized into pentamers, is stable against the thermal challenge, and folds into a beta structure . The tail, is flexible and folds mainly into loops and turns. This protein modulates the decondensation of sperm chromatin due to its ability to interact with and bind basic proteins, specifically histones H2A-H2B and sperm specific basic proteins (SPs). The poly Glu tract is believed to be critical in this interaction. NP's hyperphosphorylation during the maturation of oocytes into eggs, enhances its chromatin decondensation activity. We have found by mass spectrometry that oNP and eNP have an average number of phosphates group per protein monomer of 3 and 7-10 respectively. We have studied the interactions between NP (the recombinant full length, two deletion mutants and the proteins isolated from Xenopus oocytes and eggs) and basic proteins such as the nucleosome core histones, the linker histone H1 and SPs; using different cell biology and biochemical techniques. Our data show significant differences in the characteristics of the interaction of these kinds of basic proteins with NP.

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## 6-61

### Cell-free specific labeling and structural analysis of the transcriptional regulator RcsB

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The RcsB protein is a key regulator in enteric bacteria essential for the induction of exopolysaccharide biosynthesis, regulation of cell division and expression of osmoregulated genes. A differential targeting of RcsB might be mediated through interaction with several other coactivators like RcsA, and the 14 bp RcsAB box as a specific RcsA/RcsB binding site has been identified. We report the solution structure of the DNA binding domain of the RcsB protein solved by NMR spectroscopy. The RcsA independent DNA binding of RcsB was analysed by NMR titration studies and by surface plasmon resonance measurements with a RcsAB box consensus fragment. Using an optimized cell-free expression system, the RcsB protein could be produced in high amounts and efficiently labeled with stable isotopes and with various tryptophan analogues providing an enhanced intrinsic fluorescence. Common problems of standard in vivo labeling protocols associated with toxic effects of the label precursors, reduced protein yields or low label incorporation could therefore be eliminated. We have characterized protein-protein interactions by taking advantage of the specifically labeled RcsB derivatives and could analyse the formation of different homo- and heterooligomeric complexes. We demonstrate that the RcsAB box is specifically recognized by the RcsAB heterodimer as well as by RcsB alone. However, the binding constant of RcsB alone at target promoters was approx. one order of magnitude higher compared to that of the RcsAB heterodimer. We present evidence that the obvious role of RcsA is not to alter the DNA binding specificity of RcsB but to stabilize RcsB/DNA complexes.

## 6-62

### Structural characterization and protein interactions of the surfactin thioesterase II

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Small microbial peptides like cyclosporin or the penicillins produced by a nonribosomal pathway are of high interest due to their immense potential for clinical and therapeutic applications. The peptide bioynthesis is catalysed by large multimodular nonribosomal peptide synthetases (NRPSs) via the multiple thiotemplate mechanism. A standard elongation module consists of the three domains A-T-C and is responsible for the incorporation of one amino acid residue into the growing peptide product. While A- and C-domains catalyse the activation and condensation of amino acid residues, the central thioester forming domain (T-domain) covalently binds the activated amino acid at a specific serine residue via a thioester linkage to a 4'-phosphopantetheine (4'PP) cofactor. The 27 kDa SrfTEII protein is a specific thioesterase responsible for the regeneration of misacylated thiol groups of 4'PP cofactors attached to T-domains. SrfTEII is associated with the biosynthesis of surfactin, one of the most potent biosurfactants. We have started to solve the solution structure of SrfTEII by NMR spectroscopy and the chemical shift resonances of the amide protons of the protein backbone could be assigned. We furthermore analysed the interaction of SrfTEII with T-domains and demonstrate the formation of a tight complex. Only holo-T-domains are recognized, indicating that the 4'PP cofactor plays an important role in complex formation. While major conformational changes of the whole protein can be observed for SrfTEII upon interaction, the T-domain is specifically recognized at a central helical region in close proximity to the 4'PP cofactor.

## 6-63

### Fluorescence studies on rhBMP-2

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Bone Morphogenetic Proteins (BMP's) are a group of proteins structurally related to the Transforming Growth Factor b (TGF-b). They have devoted during the last years a great research attention due to its involvement into the patterning of embryonic development of vertebrates. Some of these BMP's have the ability to induce ectopic bone formation, and they have shown a great potential as factors inducing bone repair. This fact has focused on them a great amount of clinical and molecular biology studies. However almost nothing is known about the basic physicho-chemical properties of these proteins. In this work we show some preliminary studies on rhBMP-2, a protein engineered in our laboratory. The protein is a homodimer with seven disulphide bonds in the overall molecule. Each of the two subunits has three of these disulphide bonds and the remaining bond links the two subunits together, being the right formation of the disulphide bonds essential in order to obtain the in vitro and in vivo reported activity. In our study we have started to characterise the influence of pH (varying its value between 3 and 10,5), chaotropic agent concentration (from 0 to 8 M urea) and ionic strength on the stability of rightly folded dimer through its fluorescence spectra. Results obtained seem to indicate that the so obtained partial unfolding of the protein is a reversible process.

## 6-64

### Photobleaching of purple membranes affects the photocycle rate constants.

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Purple membranes, extracted from Halobacterium salinarum cells, are a two dimensional crystal made by bacteriorhodopsin molecules inserted in a lipid bilayer. Purple membranes reaction to illumination is driven by the retinal molecule contained in bacteriorhodopsin. Under continous illumination in the presence of hydroxylamine purple membranes bleach and a reversible loss of crystallinity of the membrane patches occurs (Moeller et al. 2000). The efficiency of photobleaching in different mutants has been studied (Subramaniam et al., 1991). It has been showed that photobleaching of purple membranes can be achieved also by continuous yellow illumination, without the use of hydroxylamine (Dancshazy et al. 1999). Here we report results on bleaching and recovering processes showing that the rate constants of the photocycle in partially bleached membranes differ from those of native membranes.

## *Poster*
*Protein structure dynamics and functions (I)*

### 6-65

**NMR studies of neurotensin and the neurotensin receptor**

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Neurotensin (NT) is an endogenous tridecapeptide neurotransmitter found in the central nervous system (CNS) and the gastrointestinal tract. High-resolution structural studies of NT and the truncated NTR agonist analogue of neurotensin NT(8-13) in the absence of the 7-trans membrane domain (7TMD) G-protein coupled (GPCR) neurotensin receptor (NTR) have revealed that no preferred conformation exists in solution as determined by studies of NMR NOEs. NT has been expressed in E. coli using the fusion vector pGEX-6p-1 and purified using affinity chromatography and HPLC. NT(8-13) has also been produced by solid phase synthesis. Both production methods for NT and NT(8-13) have been designed to permit uniform and specific labelling with NMR visible isotopes (13C, 15N, and 2H). Natural abundance solution and solid-state NMR studies were performed on NT(8-13), and an assignment carried out on the basis of unique chemical shifts. The spectra have demonstrated the presence, in solution, of two conformers arising from the cis and trans isomerisation of the Arg9-Pro10 peptide bond, with the trans isomer being the dominant conformation (87%). It has also been determined, by ROEs, that NT(8-13) has no other preferred conformation, than the dominance of the trans isomer of the Arg9-Pro10 peptide bond, in solution. This work will permit extension to structural studies of NT and NT(8-13) bound to the NTR using solid-state NMR methods as a first direct study of a ligand in the binding site of a 7TMD GPCR.

### 6-66

**Molecular dynamics study of the stability of a beta-sheet peptide**

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Solvent and temperature are two important influences on protein stability. Beta-sheet peptides have flexible structures, which vary as a function of solvent and temperature. We have performed MD simulations of a beta-sheet peptide of 24 amino acids in explicit water-methanol solvents at several temperatures. Our simulations show that the folded structure of the peptide is stablised in increasing concentration of methanol. This result is consistent with recent experimental measurements.

### 6-67

**Water state in protein solutions under radiation**

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The aqueous solutions of bovine serum albumin (BSA) human fibrinogen in the concentration interval 10-100 mg/ml and 1-10 mg/ml correspondingly have been investigated by microwave dielectric method at a frequency 9,2 GHz. The samples were gamma-irradiated in dose of 10-150 Gy. The dependencies of real and imaginary parts of the complex dielectric permittivity of native and irradiated samples against concentration have been obtained. The values of protein hydration have been calculated. The values of real part of the complex dielectric permittivity of native samples of both proteins are linearly dependent functions of concentration and decrease with increasing one. Under influence of radiation the dielectric constant of fibrinogen solutions increases. It reaches the maximum value at dose of 50 Gy. The amount of bind water at the process decreases. It can be explained by the change of the water state in solution. The non-monotone changes of dielectric permittivity dependence against concentration of BSA solutions are observed at dose of 30 Gy. The dielectric constant increases and than slows done starting with 50 mg/ml. It can be explained by the presence of macromolecule interactions as a consequence of structural changes of protein under influence of irradiation and forming the assemblies.

### 6-68

**Disruption of an ionic interaction between helices 3 and 6 decreases thermal stability of rhodopsin**

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Rhodopsin is the prototypical G-protein-coupled receptor that functions as a photoreceptor in the vertebrate retina. Transmembrane helices 3 and 6 of G-protein-coupled receptors are connected by a network of ionic interactions at the cytoplasmic side that play an important role in maintaining the receptor in its inactive conformation. The highly conserved D/ERY motif is found at the cytoplasmic end of helix 3 in these superfamily of receptors. The E134-R135 pair in rhodopsin is involved in a hydrogen-bond interaction, and at the same time R135 is hydrogen-bonded to E247 in helix 6 and this interaction is one of the constraints that keeps the receptor inactive. Single rhodopsin mutants E247A, E247Q, and the double mutant E134Q/E247Q have been constructed by site-directed mutagenesis, expressed in COS-1 cells and immunopurified in order to investigate the effect of these charge-neutralizing mutations on receptor stability. We find that the single mutants E247Q, E247A and the double mutant E134Q/E247Q show reduced thermal stability when compared to wild-type rhodopsin. This low stability correlates with disruption of the ionic interactions of the double ion pair in helices 3 and 6 of rhodopsin in its inactive ground state. The decrease in stabilility is very similar for the three mutants, indicating no synergistic effect of mutations at 247 and 134, and suggests that a negative charge at E247, rather than at E134, may be important for receptor stability of rhodopsin inactive conformation.

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## 6-69

### New insights in firmicutes cell-wall construction

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The bacterial cell-wall peptidoglycan is a network composed of glycan strands cross-linked by oligopeptides of variable composition and length. Many species of firmicutes produce branched peptidoglycan precursors resulting from the addition of a cross-bridge to the □-amino group of L-Lys in the pentapeptide stem L-Ala-□-D-Glu-L-Lys-D-Ala-D-Ala. Such cross-bridges consist of five glycyl residues in *Staphylococcus aureus* and the sequence L-Ala-L-Ser in *Weissella viridescens*. Addition of these aminoacids is catalyzed by the femABX enzymes family, that use amino acyl-tRNAs as substrates. These enzymes are potential targets for the development of new antimicrobial agents against pathogenic and resistant bacteria. Sequence analyses show that several members of the FemABX family, including FemX of *Weissella viridescens* do not possess a coiled coil region which has been proposed to be implicated in tRNA binding. FemX catalyzes the addition of the first residue to the peptidoglycan precursors, using an alanyl-tRNA as substrate. We have recently solved the structure of FemX at 1.7 Å resolution, and a complex with a peptidoglycan precursor at 2.1 Å resolution. Our structural results bring new insights in the sequence-structure-function relationships of the FemABX protein family.

## 6-70

### Glutamate 59 is important for the 3D organization of the amino acid transporter KAAT1

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KAAT1 is a neutral amino acid transporter activated by $K^+$ or by $Na^+$. The protein shows significant homology with members of the $Na^+/Cl^-$-dependent neurotransmitter transporter super family. In this study we have analysed the role of glutamate 59 that is a conserved residue in the super family. The mutant E59G KAAT1, expressed in *Xenopus* oocytes, exhibited a reduced leucine uptake (20-30% of WT) and electrophysiological analysis revealed that E59G KAAT1 has presteady-state and uncoupled currents larger than WT but no leucine-induced currents. The activity recovery observed in E59D and E59C KAAT1 suggests a role of the negative charge in this position. The effect of the highly permeant sulfhydryl reagent NEM is consistent with a tertiary structure modification of the E59G mutant. In fact, 1 mM NEM causes a 49% inhibition of leucine uptake in the native transporter and this effect is prevented by the presence of leucine, whereas the reagent causes a much higher (82%) inhibition in the mutant E59G, not prevented by the organic substrate. The different inhibition pattern exerted on proteins having the same cysteine residues, WT and E59G KAAT1, can be explained only by a modified accessibility of the target residues due to a conformational change of the protein. Phenylglyoxal (PGO), an arginine modifier, exerts a significantly higher inhibition in the mutant E59G than in the WT protein. Since arginine residues are the same in the two transporters, this effect is consistent with a modified accessibility of arginine residues to PGO in E59G KAAT1

## 6-71

### High-resolution imaging of microtubules under physiological conditions

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In eukaryotic cells microtubules (MT) form a network spanning the cell, functioning together with other filaments as internal skeleton. MT also serve as tracks for intracellular transport by molecular motors. MT themselves are rigid tubes but their (dis)assembly is a highly dynamic process. This dynamic behavior and the motor proteins enable the cell to regulate shape, transport and cell division. Electron microscopy (EM) has been used successfully to resolve the MT structure. The MT are polymerized from α-β tubulin protein dimers, joined head-to-tail into protofilaments. 13 parallel protofilaments assemble into a MT, resulting in a hollow tube with a diameter of 25 nm and a surface structure determined by the protofilaments. Although EM can be used to very high resolution it can only image fixed and stained or frozen samples. This prohibits the observation of dynamic processes in physiological conditions. We found that by using Scanning Force Microscopy (SFM) in liquid at room temperature it is possible to study non-deformed MT with a height of 25 nm. The applied force appeared critical for respecting the structural integrity of the sample; the MT were destroyed using forces exceeding 300 pN. The single protein resolution resolved internal structures in the MT, like the protofilaments (figure). Unexpectedly, also several types of point defects in the MT were found, like holes probably caused by the absence of 2 tubulin dimers. Furthermore, we explored the capability of the SFM to study the interaction between MT and motor proteins, such as kinesin.

## 6-72

### A cd investigation of conformational change in elastase following complexation with $\alpha_1$-proteinase inhibitor.

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The circular dichroism (CD) spectrum of porcine pancreatic elastase in complex with $alpha_1$-proteinase inhibitor ($alpha_1$-PI), a member of the "serpins" family, was calculated by subtracting the CD spectrum of the proteolytically cleaved inhibitor from that of the elastase-$alpha_1$-PI complex. The porcine pancreatic elastase (PPE) undergoes a moderate secondary structure change : the beta-structure is partially disordered while the alpha-helix content is poorly affected. In contrast, its tertiary structure undergoes a significant structural loosening upon complexation. These alterations have been compared to those observed in chemical and thermal unfolding of free elastase. Inhibitor-bound elastase and the chemical denaturation intermediate of free elastase share secondary but not tertiary structural features. In the serine proteinase, the conformational changes induced by the inhibitory complexation process might be sequential. The secondary structure change might reversibly occur in the primary steps, while the complex is non-covalent. The tertiary structure change, probably resulting from the docking of the enzyme in the body of the serpin, after the translocation step, might be concomitant with the irreversible conformational change of the reactive loop of the inhibitor. Results are consistent with a deformation of the active site (catalytic triad) of the complexed elastase, explaining why the ester bond linking elastase and serpin is resistant to hydrolysis, in the covalent complex. On the other hand, the thermal stability of the complexed proteinase is increased, probably due to protein-protein interaction.

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## 6-73

### Crystal structure of the *lactococcus lactis* fpg protein bound to an abasic site containing dna

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The formamidopyrimidine-DNA glycosylase (Fpg, MutM) is a bifunctional base excision repair enzyme (DNA glycosylase/AP lyase) that removes a wide range of oxidized purines, such as 8-oxoguanine and imidazole ring-opened purines, from oxidatively damaged DNA. The enzyme catalyses the hydrolysis of the base-sugar bond (glycosylase activity) and then, cleaves the DNA chain at 3' and 5' sides of the resulting abasic site (AP lyase activity). We have crystallized complexes between the *L. lactis* Fpg and duplexes DNA containing AP site analogues using wild type and mutant proteins. High resolution experiments were conducted with the best crystals using synchrotron radiation (BM30, ESRF) and the structure of a non-covalent complex between the *L. lactis* Fpg and a 1,3-propanediol (Pr) site analogue-containing DNA has been solved. Fpg consists of two globular domains. The C-terminal domain contains two DNA binding motifs: a zinc finger and an helix-two turns-helix motif (H2TH). Through an asymmetric interaction along the damaged strand and the intercalation of the triad (M75/R109/F111), Fpg pushes out the Pr site from the DNA double helix, recognizing the cytosine opposite the lesion and inducing a 60° bend of the DNA. Crystallizaton and preliminary X-ray crystallographic study of a complex between the *Lactococcus lactis* Fpg DNA-repair enzyme and an abasic site containing DNA, Karine Pereira de Jesus, Laurence Serre, Nadege Hervouet, Véronique Bouckson-Castaing, Charles Zelwer and Bertrand Castaing, 2002, *Acta Cryst.*.Crystal structure of the *Lactococcus lactis* Fpg protein bound to an abasic-site containing DNA, Laurence Serre, Karine Pereira de Jesus, Serge Boiteux, Charles Zelwer and Bertrand Castaing, 2002, *EMBO J.*

## 6-74

### Role of mutation y49f of human glutathione transferase p1-1 in the binding of glutathione and s-hexylglutathione

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Glutathione transferases (GSTs, EC 2.5.1.18) are a family of dimeric detoxification enzymes, which catalyze the conjugation of glutathione (GSH) to a variety of endogenous and exogenous electrophiles. The human pi class GST (hGST P1-1) is the most widely-distributed and the most abundant isoenzyme. Some studies have implicated this enzyme in the development and resistance of tumors toward commonly used anticancer drugs. Certain studies indicate that several residues such as Cys 47 and Tyr 49, which are located between helices $\alpha$4 and $\alpha$5 of the adjacent subunit, could participate in the intersubunit communication. The thermodynamic of binding of both the GSH and the competitive inhibitor S-hexylglutathione to Y49F of hGST P1-1 has been investigated by isothermal titration calorimetry and fluorescence. Calorimetric measurements indicated that the binding of these ligands to both, Y49F mutant and wild-type enzyme is enthapically favourable and entropically unfavourable over the temperature range studied. The affinity of these ligands to mutant Y49F is lower that those for wild-type enzyme, entropy change being the main contribution at this difference. Calorimetric titrations in several buffers with different ionization heats indicate a release of protons in the formation of complex Y49F/GSH. Probably, the thiol group of GSH releases protons to buffer media during the binding, and a group with $pK_a$ low is responsible for the uptake of protons at this pH. The temperature dependence of the free energy of binding, $\Delta G^o$, is weak because of the enthalpy-entropy compensation caused by a large heat capacity change.

## 6-75

### Influence of hydration and cation binding on parvalbumin dynamics

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Due to structural characteristics, parvalbumin exerts a major role in intracellular $Mg^{2+}$ and $Ca^{2+}$ concentration regulation during the muscular contraction -relieving cycle. This structure-function relationship being established, we are investigating the structure-dynamics-function relationship to take into account the protein dynamics. The influence of hydration on the internal dynamics of a typical EF-hand calciprotein, parvalbumin, has been investigated by incoherent quasi-elastic neutron scattering (IQNS) and solid-state $^{13}C$ NMR spectroscopy using the powdered protein at different hydration levels. Selective motions are apparent by NMR in the ten nanoseconds time-scale at the level of the polar lysyl side-chains (externally located), as well as of more internally located side-chains (from Ala and Ile). IQNS monitors diffusive motions of hydrogen atoms in the protein from a fraction of picosecond to a few nanoseconds. Hydration-induced dynamics at the level of the abundant lysyl residues mainly involves the ammonium extremity of the side-chain, as shown by NMR. The combined results suggest that peripheral water-protein interactions influence the protein dynamics in a global manner. There is a progressive induction of mobility at increasing hydration from the periphery toward the protein interior. Influence of the nature of the cation on parvalbumin dynamics is also discussed.

## 6-76

### Molecular dynamics study of protein-oligosaccharide interaction mechanisms in chitinases

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Protein-carbohydrate interactions, essential in many biomolecular recognition events, are investigated by extended molecular dynamic (MD) simulations with explicit water. The aim is to obtain atomic level information on the binding and to gain a deeper understanding of the factors determining the interactions between an oligosaccharide and a protein. The study concentrates on 42kDa chitinase from *Trichoderma harzianum* containing an extended binding site providing a number of strong and specific interactions with up to 6-7 sugar units. Since these interactions come from a limited number of loops, the substrate binding specificity can be altered by locally directed saturation mutagenesis. While experimentally determined three-dimensional structures were not available for the enzyme of interest, structural models were constructed based on the known structures of homologues. Experimentally determined sugar-protein complex structures of related chitinases were used in the initial simulations to evaluate the suitability of the force field parameters and simulation procedures. Classical MD (Gromacs) with a conventional force field and with a soft-core potential [1] is used to explore the conformational space of the chitinase loops and to study the functional behavior of the N-acetylglucosamine oligosaccharides and their derivates. Trajectories obtained from the simulations are used in analyzing the binding, especially the hydrogen bonding and hydrophobic interactions occurring via N/O-acetyl or O-methyl groups. When available, results from nuclear magnetic resonance spectroscopy are used to support and direct the computational studies and mutagenesis work.[1] Tappura et al., J. Comput. Chem. 21 (2000) 388 and Proteins 44 (2001) 167.

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## 6-77

### Mechanism of activation of calmodulin-dependent kinase I by calmodulin

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The crystal structure of the autoinhibited form of CamK-I(1-320) [1a06.pdb] shows that the autoregulatory sequence (~284-320) interacts with and distorts the substrate peptide- and ATP-binding domains which contribute to the active site of the enzyme. Activation requires that these multiple interactions are relieved by the binding of $Ca_4$CaM to its target sequence (298-320) implying the creation of the binding sites in an extended structural process. We investigated the activation mechanism by characterising the kinetic and thermodynamic properties of the complexes formed by calmodulin with the intact kinase and with the calmodulin target peptide (CKIp: kinase residues 295-318).

Binding of calmodulin to CKIp ($K_d$~1 pM) is 30000-fold stronger than to the kinase ($K_d$~30 nM). The kinetics follow an unexpectedly simple bimolecular mechanism. Furthermore, fluorescent labels attached to either the N- or C-terminal domain of calmodulin report essentially the same kinetics. Compared to the intact kinase, binding of the peptide is ~1000 fold faster, and dissociation ~30 fold slower, consistent with the observed in affinity difference. Chelator-induced dissociation of $Ca^{2+}$ from the $Ca_4$-Cam-Kinase/CKIp complexes may be described by a multi-step mechanism, involving an intermediate species, $Ca_2$-Cam-kinase/CKIp. Given the complex structural changes thought to be necessary for activation we find that the binding kinetics are unexpectedly simple, lacking any evidence (to date) of an isomerisation step reflecting the adoption of the activated conformation of the kinase. NH is supported by the Leopoldina Foundation, Halle, Germany.

## 6-78

### Insertion of a wheat protein in lipid monolayers : topological and morphological aspects

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Puroindolines are cationics (pHi=11) wheat proteins of 13 kDa. Two isoforms are found : puroindoline-a (Pin-a) and puroindoline-b (Pin-b). This work is concerned by Pin-a, which contains five disulphide bonds and a tryptophan-rich domain. Although her biological function is not clearly established, this protein is very interesting from a technological point of view, due to his foaming properties even in presence of lipids. However, questions remains concerning the kind of interaction between Pin-a and lipids. To get a better understanding of interfacial properties of Pin-a, we have studied her adsorption in lipid monolayers by surface pressure measurements, Confocal and Atomic Force Microscopy. The monolayers were transferred on mica subtracts by the Langmuit-Blodgett method. The lipids (DPPC and DPPG) were chosen in order to study the effect of their physical state and charge on Pin-a adsorption. The interfacial pressure of the lipid monolayer was stabilized to 15mN/m before the injection of protein in the sub-phase. The phase separation at 15mN/M was observed for both lipids, showing domains of rather homogeneous size (~ 25µm). The injection of protein in the subphase results in an increase of the surface pressure and a modification of the repartition of the condensed liquid phase domains into expanded liquid phase. Coupling both microscopies allows to show the influence of the charge and of the physic state of lipids on the interactions protein-lipidic film. The dense network formed by the protein and the induced lipid reorganization could explain her foams stabilization properties, even in presence of lipids.

## 6-79

### Mechanical coupling via the membrane fusion SNARE protein syntaxin-1A: a molecular dynamics study

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SNARE trans-complexes between membranes likely promote membrane fusion. For the t-SNARE syntaxin-1A involved in synaptic transmission, the secondary structure and bending stiffness of the 5-residue juxtamembrane linker are assumed to determine the required mechanical energy transfer from the cytosolic core complex to the membrane. These properties have here been studied by molecular dynamics and annealing simulations for the wild type and a C-terminal-prolongated mutant within a neutral and an acidic bilayer, suggesting linker stiffnesses above 1.7, but below 50 x cal mol^(-1) deg^(-2). The transmembrane helix was found to be tilted by 15 deg and tightly anchored within the membrane with a stiffness of 4-5 kcal mol^(-1) Ang^(-2). The linker turned out to be marginally helical and strongly influenced by its lipid environment. Charged lipids increased the helicity and H3 helix tilt stiffness. For the wild type, the linker was seen embedded deeply within the polar region of the bilayer, whereas the prolongation shifted the linker outwards. This reduced its helicity and increased its average tilt, thereby presumably reducing fusion efficiency. Our results suggest that partially unstructured linkers provide considerable mechanical coupling; the energy transduced cooperatively by the linkers in a native fusion event is thus estimated to be 3-8 kcal/mol, implying a two to five orders of magnitude fusion rate increase.

## 6-80

### Effect of ionic strength on the structure, dynamics and activity of horse liver alcohol dehydrogenase

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The room temperature tryptophan phosphorescence (RTTP) method was employed to investigate the effect of ionic strength created by KCl on the slow internal dynamics (ID) of horse liver alcohol dehydrogenase (LADH). It has been shown that the increase of KCl concentration in the range of 0 - 1.0 M lead to the decrease of average RTTP lifetime values from 750 ms to 590 ms. The observed RTTP quenching indicates a significant increase in slow ID of LADH in the vicinity of Trp-314. The effect of the protein ID labilisation is in good agreement with the loss of the enzyme activity by 60% at the increase of KCl concentration. The dependencies of RTTP lifetime and LADH enzyme activity on the KCl concentration have similar shapes indicative a functional significance of changes of the protein slow ID. To study the effect of ionic strength on the fast (nanosecond) ID and conformation of LADH the fluorescence polarization and position of maximum of tryptophan fluorescence spectrum were studied. It has been established that at increase of KCl concentration from 0 to 1 M the fluorescence polarization and position of maximum of tryptophan fluorescence spectrum remain unchanged. The data demonstrate that the fast ID and conformation of LADH do not change at the KCl concentration 0 - 1 M. The obtained experimental results evolve the unknown mechanism of enzymatic activity regulation by ionic strength via changes of the protein slow ID. This work was supported by BRFFR, Grant B02R-077.

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## 6-81

### Rational crystallization: the bovine gammaE crystallin

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Crystallins are eye lens structural proteins distributed into two families, alpha- and beta-gamma-crystallins. The crystallin interactive and associative properties determine lens transparency and opacities. Gamma-crystallins are 20kD monomers, and the seven members of the family (gamma A-F and S) share between 50 and 80% of sequence identity. Their physico-chemical properties and functions within the lens are, however, different. Several 3D structures have been determined that present similar folds yet local rearrangements. The bovine gammaE, however, was missing. Since crystallization requires attractive interactions in solution, we used Small Angle X-ray Scattering to analyse the gamma-crystallin solution properties as a function of the environment, pH, ionic strength, type of salt, temperature and PEG addition (1). At low ionic strength, attractive interactions are observed at the isoelectric point (pI). Thanks to them, gammaB and gammaD are easily crystallized around pH 7. Yet, the stronger attraction observed with gammaE is responsible for phase separation and precipitation instead of the desired crystallization. Therefore, another strategy was used. The gammaE was concentrated at pH 4.5, where the interactions are repulsive. Then salt, or polyethylene glycol, or both were added to gently turn the repulsive interactions into attractive ones. Crystals were obtained in all tested conditions. Data were collected at ESRF at 1.65 Å resolution and the structure solved by molecular replacement. An unusual stacking of arginines at the interface between domains might contribute to the special gammaE crystallin properties. (1) Tardieu A., Bonneté F., Finet S. & Vivarès D. (2002) *Acta cryst.* D58, 1549-1553

## 6-82

### Structural characterization of a fluid lipid bilayer supported on a polymer cushion by electrochemical techniques

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In this work, we show that a lipid bilayer tethered to a polyethylene-glycol (PEG) cushion chemically grafted to porous alumina is a suitable model for electrochemical studies of membrane proteins. The model membrane mimics thermo-dynamical proprieties of biological membranes by restoring an aqueous compartment between the bilayer and the solid support (porous alumina). Electrochemical monitoring of the amount of ubiquinone (coenzyme Q10 or UQ10) and of its lateral mobility in the supported bilayer demonstrates the presence of a homogeneous, continuous and fluid bilayer at a large scale (more than 5 cm2). Electrochemical monitoring of the micro-viscosity in the bilayer shows that the supported bilayer is free from interactions with the solid support. Experimental values of micro-viscosity follow a model of free energy activation with a rupture at the lipid main transition temperature. Using a geometrical approach, electrochemical monitoring is used to follow the decrease of the porosity of the alumina support due to the formation of the tethered bilayer inside the pores. It reveals the existence of an aqueous subcompartment whose thickness can be measured. Using polymer chains with different molecular weights, we show that the thickness of the aqueous compartment is equal to the Flory radius of the polymer chains. The self-assembled bilayer is a suitable medium to incorporate membrane proteins and to study their catalytic properties.

## 6-83

### Inhibition of the alzheimer β-peptide aggregation by transthyretin studied by fluorescence correlation spectroscopy

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Familial amyloid polyneuropathy is an inherited and neurological disease where the plasmaprotein transthyretin (TTR) is precipitated along peripheral nerves as amyloid. It can also be deposited in the vitreous of the eye but brain deposition is a rare event. TTR is a rather abundant protein in plasma where it is bound to its ligands thyroxine and retinol-binding-protein. In the cerebrospinal fluid of the brain, TTR is the most common protein. It is of great interest for us to study whether TTR is important for the formation and deposition of amyloid fibrils in Alzheimer´s disease. We are keenly interested to test whether TTR can inhibit/desolve the aggreagation of the amyloid β-peptide. The fibrillar deposit of Alzheimer amyloid β-peptide (A□) in brain is a key step in Alzheimer's disease. Studying of A□ polymerization and its inhibition is a potential strategy for drug design relevant for Alzheimer's disease. Fluorescence correlation spectroscopy (FCS) is a powerful biophysical tool for studying molecular interactions of biological importance (Rigler, J. Biotech. 41 (1995) 177-186). Using FCS we have for the first time followed the A□ polymerization in real time, detecting monomers, oligomers and large aggregates simultaneously (Tjernberg et al., Chem. Biol. 6 (1999) 53-62). Very recently, we found that the aggregation the A□ peptide could be inhibited by the addition of equimolar concentrations of TTR. These results are one step forward for a drug design relevant for Alzheimer's disease.

## 6-84

### Binding of blue copper proteins to redox partners – structural control of binding as a function of redox state

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Blue copper proteins are small copper proteins transporting electrons between redox partners that are often big membrane bound protein complexes. Recent investigations have shown that in several cases the binding of the blue copper protein to its redox partner is strongly dependent on the redox state of the proteins. By substituting the copper ion by radioactive cadmium or silver it is possible to study the structure of the metal site by the technique of Perturbed Angular Correlations. These studies show that both plastocyanin and amicyanin have dynamic metal sites where binding to photosystem I or methylaminedehydrogenase respectively constrains the metal site to one conformation. The experiments further suggest that the metal site structure plays an important role in controlling the binding as a function of the redox state of the copper ion. Danielsen E, Sheller HV, Bauer R, Hemmingsen L, Bjerrum MJ, Hansson Ö, (1999) "Plastocyanin binding to photosystem I as a function of charge-state of the metal ion: The effect of metal site conformation". Biochemistry, 38(35), 11531-11540. Jørgensen LE, Ubbink M, Danielsen E, "Amicyanin metal site structure and interaction with MADH". To be submitted

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## 6-85

### Acid pH promote molten globule state of frutalin: an oligomeric lectin

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The elucidation of the "folding problem" includes the identification of folding pathway, as well as, the partially folded states named *molten globule* (MG). The interest in this subject has been focus on its physiological role, in addition to its importance in several degenerative diseases. The folding studies using oligomeric proteins, particularly in β-fold, are rare and can provide an insight regarding the tertiary and quaternary interactions. In this sense, our group has been studying the refolding of frutalin, a D-Galactose binding lectin, quite stable within chaotropic agents and temperature having reversibility of these forms (Campana et al, EJB, 269 (3): 753-758, 2002). In present work, the stability of the frutalin was studied at several pH values in the presence and absence of D-Galactose, its sugar binding. Homogeneous frutalin native form, submitted at large pH range (2.0 to 12.0), was monitored by steady-state fluorescence and anisotropy, circular dichroism (CD), size exclusion chromatography methods and hemagglutination activity. Frutalin showed a high stability from pH 5 to 8, showed by CD, fluorescence and biological activity. The sugar binding presence showed stabilization of the tetrameric forms at pH 9 and 10. In addition, at acid values, typically below pH 4, molten globule states were found, besides having no reversibility. In conclusion, was demonstrated that at acidic pH, oligomeric β-fold protein, could adopt a molten globule state, being susceptible to aggregation, as found in protein conformational diseases.

## 6-86

### Conformational differences in solution between glutamate receptor ligand binding domain complexed with agonists and antagonists as revealed by SAXS

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The glutamate receptor ion channels (iGluR) are responsible for most excitatory synaptic signaling in the central nervous system. Their ligand binding domain are structurally homologous to the bacterial periplasmic binding proteins for which the two lobes of the protein are separated by a binding cleft that is open in the empty state and closed in the ligand-bound state (Venus flytrap mechanism). Spectroscopic studies of GluR-LBD revealed a conformational change, but no decrease of Rg was detected by Small Angle X-ray Scattering (SAXS) upon substrate binding. Recently, a series of crystal structures revealed the cleft closure associated with agonist binding. To resolve the discrepancy between crystal and SAXS results, SAXS experiments were performed using both the core GluR2 construct (S1S2J) used in crystallographic studies and the extended construct that includes the peptides that link the core domain to the N-terminal and transmembrane domains in the intact subunit used in the previous solution scattering work. Measurements performed on S1S2J with agonist or antagonist yield scattering patterns very close to those calculated from the crystal structures, while a small difference is observed with the apoprotein, which seems to be in equilibrium between both extreme conformations. Measurements on different preparations of the full-length construct reproducibly exhibit small but significant differences best visible on the distance distribution functions. Two conformations can be characterized

## 6-87

### Equilibrium fluctuations in myoglobin, lysozyme and reaction center

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Angle dependencies of total and inelastic intensities of Rayleigh scattering of Moessbauer radiation were measured for myoglobin, lysozyme and membrane protein photosynthetic Reaction Centre RC for different hydration degrees. Treating of data for hydrated proteins approves existence of segmental motions (alpha helices) for myoglobin (alpha helices and beta sheets) for lysozyme as well as of individual motions. Further hydration increases mean square displacements for both types of intraglobular motions for these proteins while long range correlated motions remain nearly the same as for dehydrated sample. Correlation times of intraglobular motions for lysozyme are of order of nanoseconds or less. Correlation times of segmental motions in myoglobin are of order of hundreds nanoseconds. Results of study of radial distribution function deduced by Fourier transform from diffuse x ray measurements together with RSMR data allow to conclude that water during hydration of proteins competes with intramolecular hydrogen bonds loosens the protein and increases internal dynamics. Analysis of auto and cross correlation functions of bending fluctuations of alpha helices of large domain of lysozyme performed by molecular dynamics allows to come to final conclusion that exactly the difference in architecture of myoglobin (alpha protein) and lysozyme (alpha plus beta protein) but not the SS bonds in lysozyme macromolecule is the real reason of different structural dynamics of these proteins The first data for RC show some difference as compare to myoglobin and lysozyme.

## 6-88

### The E1/E2 distribution of states of the fully-active Na/K-ATPase probed by voltage clamp fluorometry

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It was shown recently that the Na,K-ATPase single-cysteine mutant N790C expressed in Xenopus oocytes forms a molecular sensor complex upon covalent attachment of tetramethylrhodamine-maleimide, whose fluorescence intensity follows the redistribution between the E1 and E2 conformational states in voltage clamp fluorometric experiments [Geibel et al. (2003). PNAS 100:964-969]. Voltage jumps carried out under Na/Na exchange conditions (extracellular [K+] = 0) induce fluorescence changes whose properties match those of the transient currents originating from Na translocation steps during the E1P-E2P conformational transition. Using this method we investigated the effects of Na, K, NMDG, quarternary ammonium derivatives and pH on the voltage dependence of the E1P-E2P conformational change. The voltage dependence of the stationary fluorescence intensity follows a Boltzmann distribution whose midpoint is shifted towards positive potentials with increasing Na concentration. The absolute fluorescence changes upon defined voltage jumps are maximal at pH 7.4 and the V0.5 value is shifted towards positive potential upon acidification. NMDG and quarternary ammonium derivatives differentially affect amplitudes and voltage dependence of the voltage jump-induced fluorescence changes. Furthermore, voltage jump-induced fluorescence changes can be recorded at sub-saturating K concentrations (i.e. turnover conditions) either in presence or absence of extracellular Na. The Na-dependent differences in relaxation kinetics at a given K-concentration yields information about voltage dependence and time course of K-dependent reaction steps under turnover conditions of the fully active enzyme.

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## 6-89

### The effect of induced 50Hz electromagnetic field on the protein structure of Escherichia coli and Staphylococeus aureus bacteria and their biological response

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The changes in the molecular structure of water soluble protein (wsp) extracted from E.coli. and S.aureus due to exposing to 25 gauss of electromagnetic field EMF were investigated after exposure period 24 hrs respectively. This job was achieved through measuring each of the dielectric relaxation and the subunit molecular weight of wsp of each microorganism using SDS polyacrylamide gel electrophoresis. The effect of such field on each of the growth rate, the antibiotic sensitivity and pathogenesity tests for exposed and unexposed bacteria were carried out. The growth rate was measured before and after exposing of both organisms to different electromagnetic field strengths 10, 20, 25 gauss for 4, 8, 12 and 24 hrs exposing periods respectively.Both of the antibiotic senstivity and bathogensity test were performed for the highest field strength and period. The results indicated remarkable changes in the molecular structure of the extracted protein molecules of the exposed bacteria. Also, there was sharp reduction in the number of protein bands and decrease in its protein content. The results, also revealed a decrese in the growth rate of the exposed bacteria. The maximum decrease was achieved after exposing dose 25 gauss and exposure period 24 hrs. Also there are increasing in the sensitivity of the exposed bacteria to certain antibiotics and decreasing in their virulence effect. The mortality rate recorded after the injection with exposed and unexposed E.coli ware 60% and 90% respectively.Also the cutanceous disease and abscesses caused by St .aureus disappeared after the injection with the exposed bacteria.

## 6-90

### Thermodynamics of heme binding to HasAsm hemophore

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$HasA_{SM}$ secreted by the gram-negative bacterium *Serratia marcescens* belongs to the hemophore family. Its role is to take up heme from host heme carriers and to shuttle it to specific receptors. Heme is linked to the $HasA_{SM}$ protein by an unusual axial ligand pair: His32 and Tyr75. The nucleophilic nature of the tyrosine is enhanced by the hydrogen bonding of the tyrosinate to a neighboring histidine in the binding site: His83. We used isothermal titration microcalorimetry to examine the thermodynamics of heme binding to $HasA_{SM}$ and showed that binding is strongly exothermic and enthalpy driven: $\Delta H$=-105.4 kJ/mol, $T\Delta S$=-44.3 kJ/mol. We used displacement experiments to determine the affinity constant of $HasA_{SM}$ for heme ($Ka=5.3x10^{10}$ $M^{-1}$). This is the first time that this has been reported for a hemophore. We also analyzed the thermodynamics of the interaction between heme and a panel of single, double and triple mutants of the two axial ligands His32 and Tyr75 and of His83, to assess the implication of each of these three residues in heme binding. We demonstrated that, in contrast to His32, His83 is essential for the binding of heme to $HasA_{SM}$, even though it is not directly coordinated to iron, and that the Tyr75/His83 pair plays a key role in the interaction.

## 6-91

### Antifolding activity of the SecB chaperone is essential for secretion of HasA, a quickly folding ABC pathway substrate.

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We have previously shown that SecB, the ATP-independent chaperone of the sec pathway is required for the secretion of the HasA hemophore from *Serratia marcescens* via its type I secretion pathway, both in the reconstituted system in *Escherichia coli* and in the original host (1, 2). We have proposed a model whereby the N-terminus of HasA interacts in a SecB dependent fashion with the ABC protein of the transporter, allowing an efficient interaction between the C-terminal secretion signal with the transporter. The refolding of apo-HasA after denaturation with GdmCl was followed by stopped-flow measurements of fluorescence both in the absence and presence of SecB. In the absence of SecB, HasA folds very quickly with one main phase ($70s^{-1}$) accounting for 95% of the signal. SecB considerably slows down HasA folding. At stoichiometric amounts of SecB and HasA, a single phase ($0.03s^{-1}$) is observed. Two double point mutants of HasA were made, abolishing two hydrogen bonds between N-terminal and C-terminal side-chain residues. In both cases, the mutants did not display alteration of secondary and tertiary structure and were fully functional. Refolding of both mutants was much slower than that of wild-type HasA and they were secreted essentially independently of SecB. We conclude that SecB has mainly an unfolding function in the HasA ABC secretion pathway. Delepelaire P, Wandersman C. (1998) *EMBO J.* 17(4):936-44. Sapriel G, Wandersman C, Delepelaire P. (2003) *J Bacteriol* 185(1):80-8.

## 6-92

### Structural Study by 1H NMR of ErgToxin, a specific blocker of HERG channels

Frenal [1], K., Wecker [1], K., Possani [2], L. Wolff [1], N., Delepierre [1], M.
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Recently, a peptide that inhibits specifically with a high affinity ERG (*ether-a-go-go* related gene) channels, was isolated from the venom of the *Centruroïdes noxius* Mexican scorpion: the Ergtoxin (ErgTx) (1). The ERG channels, mostly expressed in heart, are voltage-dependant potassium channels impaired in the $I_{Kr}$ inward rectification current which plays a role in maintaining cardiac electrical stability. They are also well expressed in peripheral sympathetic ganglia, brain, and tumor cells. Erg gene is a member of the eag super family of genes encoding $K^+$ channel with six transmembrane domains and a highly conserved P-region that conforms the ion pore in the tetrameric organization of the channel. Mutations on the erg gene are responsible for the chromosome 7-linked long QT syndrome (LQT2) that may cause syncope and sudden death resulting from arrhytmias and ventricular fibrillation. In order to gain information about the topology of HERG channels, the structural study of Erg has been performed by NMR. Erg is a 42 residues short toxin with four disulfide bridges with no sequence homology with other $K^+$ channels toxins. The ErgTx $^1$H NMR assignments, secondary structures and global fold are reported here. On the basis of structure and sequences of □-KTX toxins, we propose a binding motif common to the gating modifier of ERG channels. (1) Gurrola GB, Rosati B, Rocchetti M, Pimienta G, Zaza A, Arcangeli A, Olivotto M, Possani LD, Wanke E. (1999) *Faseb J* **13**, 953-62.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-93

### A XAS study of the geometrical arrangement of Cu(II)-binding octarepeat peptides

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The interest about the group of neuro-degenerative diseases belonging to the so-called "Prion diseases", is growing day-by-day due to their strong economical and sociological impact connected with the development of transmissible bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jacob disease (CJD) in humans. Among others, a very important point, which is still waiting for an answer, is to clarify the functional role of the native cellular prion protein ($PrP^C$). There exist indirect evidences attributing to $PrP^C$ a role in copper metabolism and transport. Metal ions are bound to the N-terminus region where the amino acidic sequences of both human and bovine $PrP^C$ is characterised by the presence of five and four repeated sequences respectively, each composed of eight amino acidic residues (octarepeat). None of the many spectroscopic techniques used so far provides a direct determination of the geometrical structure around Cu(II). This geometry can be instead directly investigated by X-ray absorption spectroscopy (XAS). We used XAS to clarify the binding of $Cu^{2+}$ ions to synthetic peptides whose amino acidic sequences correspond to sections of the N-terminal domain of the mature PrP. We collected XAS spectra (at the EMBL-DESY Hamburg (Ge) facility) on $Cu^{2+}$-peptide complexes made of 1, 2 and 4 octarepeats respectively. The analysis of the XAS spectra clearly shows that $Cu^{2+}$ geometrical environment is different between the 1- and 2- octarepeat peptides on one side and the 4-octarepeat peptide in the other

## 6-94

### NMR conformational studies of oligosaccharide and peptide mimics of the *O*-specific polysaccharide of *Shigella flexneri* 5a.

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The aim of the work is to develop synthetic chemically defined vaccines against shigellosis, a dysenteric syndrome caused by a Gram-negative enterobacterium, *Shigella.* The bacterial *O*-specific polysaccharide (*O*-SP), a component of their lipopolysaccharide, is an essential virulence factor and a critical antigen for host immunity. The chemically defined vaccine approach consists in using simple molecules, like oligosaccharides or peptides, able to mimic the *O*-SP. On the model bacterium *S. flexneri* 5a, different synthetic oligosaccharides representative of carbohydrate epitopes and immunogenic peptide mimics, selected by screening phage-displayed peptide libraries with protective antibodies, are available. Optimization of the mimics of the antigenic polysaccharide is supported, in part, by a comparative study of their solution conformations with that of the native carbohydrate epitopes. Therefore, a structural study of the conformations of the synthetic pentasaccharides representative of the *O*-SP of *Shigella flexneri* serotype 5a (1,2,3) and immunogenic peptide mimics has been performed. Then, the conformation of oligosaccharides and peptides in interaction with protective monoclonal antibodies have been obtained by means of Transferred Nuclear Overhauser Effect experiments while the binding epitope determined with Saturation Transfer experiments

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## 6-95

### Biophysical studies show the smaller subunit of m-calpain to contain a lipid interactive α-helical segment

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m-Calpain, is a medically important heterodimeric protease whose activation appears to involve the membrane interaction of a segment in the enzyme's smaller subunit, which we have previously predicted to form a lipid interactive, oblique orientated α-helix. Here, FTIR conformational analysis shows a peptide homologue of this segment to adopt high levels of α-helical structure (> 60%). At a surface pressure mimetic of naturally occurring membranes (30 mN $m^{-1}$), the homologue interacted strongly with anionic and zwitterionic monolayers, inducing surface pressure changes of the order of 5 mN $m^{-1}$. The peptide induced similar pressure changes in monolayers formed from POPC/POPS (10:1 molar ratio) whilst neutron lamellar diffraction studies showed the peptide to interact with the acyl chain region of membranes of similar lipid composition. These results clearly show that the peptide is able to form a lipid interactive amphiphilic α-helix and able to penetrate deeply into the membrane lipid core, strongly supporting our structural prediction and supporting a role for membrane interactions in the modulation of enzymatic activity.

## 6-96

### The N-terminal region of the Barley Yellow Dwarf Virus Movement Protein shows the potential to form a lipid interactive oblique orientated α-helix.

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The Barley Yellow Dwarf Virus Movement Protein (BYDV-MP) is of agronomical importance, having been shown to interact with the nuclear membrane of host plant cells, promoting viral entry into the nucleus. Extended hydrophobic moment plot analysis showed BYDV-MP to possess an N-terminal segment with $< \mu H > = 0.33$, amphiphilicity consistent with that of segments known to form lipid interactive oblique orientated α-helices, such as the HA2 influenza peptide. Helical wheel analysis showed the BYDV-MP α-helix to possess strong structural resemblances to that of the HA2 peptide, in particular a polar face rich in glutamic acid residues. Based on the mechanism by which HA2 promotes viral cell entry, we have hypothesised that protonation ofthese residues in the BYDV-MP N-terminal segment may promote α-helix formation and membrane interaction by the segment. Consistent with this hypothesis, FTIR conformational analysis has shown that in the presence of lipid vesicles, a peptide homologue of the BYDV-MP N-terminal segment is predominantly β-sheet at pH 7 (>90%) but adopts high levels of α-helical structure at pH 5 (50%). This decrease in pH also led to a strong decrease of 10°C in the lipid phase transition temperature of anionic lipid.

## *Posters*

*- Protein structure dynamics and functions (I) -*

## 6-97

### A statistical investigation of amphiphilic properties of C-terminally anchored DD-peptidases

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A number of studies on DD-peptidases have reported these bacterial enzymes to interact with the membrane *via* C-terminal amphiphilic α-helices but experimental support for this is limited. These studies show the C-terminal interactions of DD-carboxypeptidases to involve high levels of membrane penetration, DD-endopeptidases to involve membrane surface binding and class C PBP's to involve membrane binding with intermediate properties. Here, we have characterised C-terminal α-helices from each of these peptidase groups according to their amphiphilicity ($<\mu H>$) and corresponding mean hydrophobicity ($<H>$). Regression and statistical analyses showed these variables to exhibit parallel negative linear relationships, which resulted from the spatial ordering of α-helix amino acid residues. Taken with the results of compositional and graphical analyses, our results suggest that the use of C-terminal α-helices may be a universal feature of DD-peptidase - membrane anchoring. Our results also suggest that to accommodate differences between these mechanisms, the C-terminal α-helices of each group optimise their structural amphiphilicity and hydrophobicity to fulfil individual membrane anchoring function. Our results show that each anchor type analysed requires a similar overall balance between amphiphilicity for membrane interaction, which we propose is necessary to stabilise their initial membrane associations. In addition, we present a methodology for the prediction of C-terminal α-helical anchors from the classes of DD-peptidases analysed, based on a parallel linear model.

## *Posters*

*- Protein structure dynamics and functions (II) -*

## 7-1

### Homology modeling and site-directed mutagenesis of the cardiotrophin-like cytokine reveals a key role for trp-67 in cntf receptor alpha binding

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IL-6 type cytokines act by the formation of a multimeric receptor complex including a common receptor unit, gp130. This family, containing CLC and CNTF, belongs to the four helix bundle class and bind to their receptors through three binding sites numbered from I to III. In spite of low sequence identity (16%), CLC and CNTF act through the same tripartite receptor which includes gp130, LIFRbeta and CNTFRalpha. The limiting step in CLC modeling is the alignment of the IL-6 type cytokines. Multiple alignments of (1) paralogs or (2) paralogs and orthologs yield two models of CLC differing at the CNTFRalpha binding site. Two models of the CLC-CNTFRalpha complex are built from the two putative CLC models. Analysis of the interfaces suggests that, in either case, a Trp residue (Trp-67 or Trp-169) can act as a binding hot spot. Site-directed mutagenesis of CLC is carried out to determine which Trp is involved in CNTFRalpha binding. CNTFRalpha and CLC bearing either the W67A or the W169A mutation are co-transfected in Cos-7 cells. Secretion of CLC requires CNTFRalpha binding. Western blot analysis of supernatants reveals that the W67A mutation prevents secretion of CLC whereas the W169A mutation has no effect. This indicates that Trp-67 is involved in CNTFRalpha binding. CLC Trp-67 is aligned with CNTF Trp-64 which has been shown to be a hot spot for CNTFRalpha binding. Thus, in spite of the low sequence identity between CLC and CNTF, key determinants for CNTFRalpha binding are conserved.

## 7-2

### Hydration of homopolypeptides studied by double calorimetry

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Characterization of the physical properties of water is essential in understanding the structure and function of proteins. In this work the free energy and enthalpy of hydration have been determined in a number of homopolypeptides, using a new double calorimetric method. The method allows simultaneous determination of the changes upon hydration and the amount of water sorped to the peptides. The chemical composition has a large influence on the amount of water sorped, ranging from 0.5 g water/g peptide for polyglutamic acid at a water activity of 0.8, down to 0.01 g water/g peptide for polyphenylalanine at the same water activity. Conversely, the enthalpy of sorption is rather similar for the investigated polypeptides. Thus, the enthalpy of sorped water at a moderate degree of hydration is about 47 kJ/mol lower than that in the vapour phase. It follows that the enthalpy of sorped water is only marginally lower (2-4 kJ/mol) than that of the pure liquid, and this indicates that the driving force for "binding" water to peptides is not favorable energetic interactions between water molecules and the sidechains, but rather the entropic changes that water induces in the peptides.

## 7-3

### Overexpression, purification and refolding of the human mu-opiate receptor

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Elucidation of the mechanism of activation of GPCR by neuropeptides at a molecular level is one of the major goals of structural biology and pharmacology, due to the scarce information currently available and the importance of this protein family for drug development. We are specifically involved in the enkephalin human mu opiate receptor system and one of our objectives is to determine the receptor-bound conformation of enkephalins, which is responsible for receptor activation. In order to get structural information; we have developed an efficient system for the overexpression in P. pastoris of this receptor, in a functional form (1). P. Pastoris was shown to be compatible with growth in a perdeuterated medium and 85% deuterated, functional receptor could thus be produced (2) . Perdeuteration, and more generally stable isotope labelling (13C, 15N), is of prime importance for various NMR experiments (transferred NOE, TROSY). Using a fusion protein with GFP we could optimise the expression conditions, follow accurately the yield of expression and the solubilisation/purification procedure (3,4) . Several mg of pure receptor can now be produced and purified routinely. This opens the way to the development of efficient refolding strategies and to the analysis of the folding mechanism. Several environments are currently being investigated, detergents, lipid bilayers, organic solvents, and the efficiency of folding is followed by fluorescence, circular dichroism and radioactive ligand binding experiments. (1) Talmont, F., Sidobre, S., Demange, P., Milon, A., and Emorine, L. J. FEBS Lett, 394, 268-72 (1996). (2) Massou, S., Puech, V., Talmont, F., Demange, P., Lindley, N. D., Tropis, M., and Milon, A. Journal of Biomolecular NMR, 14, 231-239 (1999). (3) Sarramegna, V., Talmont, F., de Roch, M. S., Milon, A., and Demange, P. Journal of Biotechnology, 99, 23-39 (2002). (4) Sarramegna, V., Demange, P., Milon, A., and Talmont, F. Protein Expression and Purification, 24, 212-220 (2002).

## 7-4

### Propensity of amino acids in loop regions connecting beta-strands

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Loops are regions between secondary structures of proteins they have various lengths and three dimensional configurations. It is well known that the determination of the structures of loops is a difficult problem; loops are also thought to assume important roles in molecular function and biological recognition. Therefore, analyzing the propensity of amino acids in loops should provide new insights for studying these problems. In order to calculate the propensity of amino acids in loops connecting Beta structures we used the Sloop Database of Super Secondary Fragments, the propensity in Strand-Loop-Strand classes was calculated. In this regard, all the families were searched and the loop amino acids were extracted. The propensity of occurrence of different amino acids in loop structures related to the amino acid percentage in the Swiss-Prot database was calculated. Results show that Asn is the mostly occurring amino acid in loop regions connecting Beta-Strands, followed by Gly, Asp, Ser, Thr and Pro, all other 20 amino acids show a propensity lower than one, indicating that they are not allowed in loop regions. The results presented here can be used in modeling loop structures connecting Beta-Strands in De novo designed proteins.

## *Posters*

*- Protein structure dynamics and functions (II) -*

## 7-5

### Preferential binding of two compatible solutes to the glycan moieties of Peniophora lyci phytase

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Regulation of hydration behavior, and the concomitant effects on solubility and other properties, has been suggested as a main function of protein glycosylation. In this work we have studied the hydration of the heavily glycosylated Peniophora lycii phytase in solutions (0.15-1.1 molal) of the two compatible solutes glycerol and sorbitol. Osmometric measurements showed that glycerol preferentially bind to phytase (i.e. glycerol-glycoprotein interactions are more favorable than water-glycoprotein interactions), while sorbitol is preferentially excluded from the hydration sphere (water-glycoprotein interactions are the more favorable). To assess contributions from respectively carbohydrate and peptide moieties, we compared phytase (Phy) and a modified, yet enzymatically active form (dgPhy) in which the glycans had been removed. This revealed that both polyols showed a pronounced and approximately equal degree of preferential binding to the carbohydrate moiety. This preferential binding of polyols to glycans is in contrast to the exclusion from peptide interfaces observed here (for dgPhy) and in numerous previous reports on non-glycosylated proteins. In spite of the distinct differences between peptide and carbohydrate groups, glycolysation had no effect on the stabilizing action provided by glycerol and sorbitol. Based on this it was concluded that the carbohydrate mantle of Phy must be fully accessible to the solvent, and thus that its interactions with compatible solutes has little or no effect on conformational equilibria of the glycoprotein. Other properties such as solubility and aggregation behavior, on the other hand, may be strongly modified by the favorable polyol-glycan interactions.

## 7-6

### Bio-macromolecule phase separation studied by time-resolved small angle x-ray scattering: relevance to protein crystallisation

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The interactions of biological macromolecules (hard sphere and Coulombic repulsion, Van der Waals attraction, etc) are responsible for their thermodynamic properties. In particular, it is now well documented that crystallization requires attractive interactions. Small angle X-ray scattering (SAXS), combined with numerical simulations was used to investigate the molecular interactions involved in phase separation (fluid-fluid or fluid-solid) induced by polyethylene glycol (PEG) in solutions of alpha-crystallins, one of the eye lens characteristic proteins, and Brome Mosaic Virus (BMV), an icosahedral plant virus. In both cases, the addition of PEG was able to modify the interactions from a repulsive to an attractive regime. The increase of the PEG concentration or size induces an increase of the attraction, until a phase separation is observed, with one phase enriched in macromolecules and the other in PEG. The high brilliance of ID-two (ESRF,Grenoble), coupled to a rapid camera and a stopped-flow apparatus enabled us to perform high spatial resolution and time-resolved experiments in the millisecond range. The kinetics of phase separation were studied as a function of time for different initial macromolecules and PEG concentrations and size. The polymer-induced depletion attraction leads to a fluid-fluid for the alpha-crystallins, and a fluid-solid transition for the BMV. For the BMV, the crystalline system was found to be face centered cubic. These studies have shown that the PEG-induced attractive potential is short range, which explains why PEG favours macromolecular contacts and therefore protein crystallization.

## 7-7

### Insights into the role of the L6/7 loop in SERCA1a

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SERCA1a (sarcoplasmic reticulum Ca2+-ATPase) is a membrane protein playing a central role in muscle relaxation by actively accumulating Ca2+ into the reticulum lumen. Site-directed mutagenesis studies previously highlighted the role of aspartate residues D813 and D818 located in the loop connecting the 6th and 7th transmembrane spans for ATPase activation by Ca2+. Using Ni2+-affinity chromatography we have now purified milligram amounts of the D813A-D818A mutant (ADA) overexpressed in yeast. After reconstitution into lipids, the ADA Ca2+-ATPase, at high ATP concentration, displayed a Vmax similar to that of the wild-type (WT) ATPase and a Km for Ca2+ in the micromolar range, i.e. higher than that of WT enzyme but by less than one decade. In contrast, at low ATP concentration, ATP hydrolysis by the ADA ATPase was strongly reduced at all Ca2+ concentrations. When ADA was solubilized with a mild detergent, the Vmax was also dramatically reduced. The intrinsic fluorescence changes associated with calcium binding were also monitored, for both ADA and WT reconstituted ATPases; again, the affinity of ADA for calcium was shifted by about one decade, compared to WT. In phosphorylation experiments with the ADA mutant at a low ATP concentration, we found that micromolar Ca2+ concentrations activated phosphoenzyme formation from [gamma-32P]ATP, although up to a steady-state level lower than that of WT ATPase. All these experiments suggest that the L6/7 loop plays a critical role in stabilizing the architecture of the ATPase and controls the rate of the conformational change induced by Ca2+ binding, an ATP-sensitive step.

## 7-8

### Protein conformation, hydration and water molecules dynamics. Influence of physical factors

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The conformation state and stability of a protein is determined by intramolecular factors and depend on the features of the interaction of macromolecules with solvent. The physical properties of water are sensitive to the same factors that influence protein stability, so that some connection is likely. The use of dielectric measurements in the range of bulk water relaxation allows one to investigate the protein conformation and hydration at varies conditions because the dielectric permittivity is a very sensitive parameter to the change of the water molecule state. The conformational state of proteins such as fibrinogen and albumin in aqueous solutions by microwave dielectric method at the frequency 9,2 GHz has been investigated. The dependencies of real and imaginary parts of complex dielectric permittivity of studied proteins against the temperature in the range 10-70 C have been obtained. The values of protein hydration have been calculated. The solutions of BSA in the concentration interval 5-50 mg/ml were used. The changes of dielectric permittivity for BSA are observed at doses 15 and 45 Gy. For solutions of native albumin at the temperatures 38-44 C and 54 C the change of dielectric permittivity were observed. The hydration of fibrinogen (36-45 C) raises. For native fibrinogen in the region of temperatures 52-60 C is observed predenaturation transition accompanying with rising of the hydration. This transition might be connected with structural transition in peripheral domains of fibrinogen.

## *Posters*

*- Protein structure dynamics and functions (II) -*

## 7-9

**Integrated molecular modeling tools to investigate protein–ligand interactions: "thor program"**

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A large number of data are now available from genome projects and could be now translated into structure-function relationship. The computer can be used to predict and simulate protein structures and to engineering ligands as possible drugs and function modulators. Software is available to classify and predict protein structures based on their sequence motifs and homology. Further investigation needs an integrated environment to deal with molecular modeling computational tools. We have developed a software package (THOR) in order to simulate molecular interactions between ligands and proteins. The program includes geometry optimization, stochastic folding predictions and molecular dynamics simulations, allowing for implicit or explicit representation of solvent molecules. We have worked out explicit representation to deal with lipid bilayers and full hydrated macromolecular complexes. Detailed analysis of the electrostatic field produced by macromolecules is also obtained by means of quantum mechanics calculations of multi-centered electric multipoles. We have explored these methods to investigate interactions of inhibitors with catalytic sites of important cysteine proteases of Chagas disease and of Malaria, as well as proteases and potential inhibitors of drug-resistant mutants of the HIV-1. We are simulating also the binding of specific polysaccharides to lectins and of a super-antigen enterotoxin to a T cell receptor.

## 7-10

**Solvation, stability and solubility of halophilic proteins**

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To be active, stable and soluble in high salt are major challenges facing proteins in halophilic microorganisms. Through the evolution of their sequences, halophilic proteins have, therefore, evolved specific molecular mechanisms that allow them to be both stable and soluble in the high KCl concentration of the cytoplasm. High salt has become an absolute requirement, since halophilic proteins in general unfold at low salt.
We evaluated the solvation, stability and weak inter-particle interactions of malate dehydrogenase from *H. marismortui* (*hM* MalDH) in various salt solutions, in order to probe the role of the ions and water of the solvent. *hM* MalDH adapts to its environment: its global solvation depends strongly on the salt nature [Ebel et al. *Biochemistry* 2002]. Strong (detected by crystallography) and weak binding sites for solvent ions explain the effect of salt on protein stability and auto-association [Ebel et al. *Biochemistry* 1999, Irimia et al. *J. Mol. Biol* 2003]. Attraction between proteins is observed when the composition of the solvation shell is different from the bulk, which can be understood by thermodynamic relationships. These studies explain the adaptation of halophilic proteins [Costenaro et al. *Biochemistry* 2002, Costenaro & Ebel *Acta Cryst D* 2002] and also their protocols of crystallization [Costenaro et al *J Cryst Growth* 2001].

## 7-11

**Intermolecular interactions and crystallogenesis of urate oxydase ; fundamental study and applications**

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Biological crystallization is an interdisciplinary area whose primary goal is the production of good quality crystals for structural biology. We present the crystallogenesis study of Urate oxidase, a large homotetrameric protein (Mw=128kDa,D=70A) whose structure has been previously solved at 2.05A [1]. Protein interactions in solution which govern protein crystallization were analyzed by Small-Angle X-ray Scattering (SAXS) in terms of second virial coefficient (A2). We found a close correlation between the variation of the A2 parameter as a function of PEG (PolyEthylene Glycol) addition and Urate oxidase solubility [2]. Numerical treatments coupled with SAXS experiments enabled us to determine the corresponding PEG-induced attractive pair potential, which is closely linked to the shape of the protein phase diagram [3]. In parallel, we have determined, as a function of PEG addition, the protein experimental phase diagram: the solubility and the liquid-liquid demixion curves. Different crystal morphologies were observed depending upon the location in the phase diagram. We performed crystal growth studies to investigate the influence of the liquid-liquid phase separation on the crystallization and to discriminate the different crystal forms observed. All these studies allowed us to get good resolution crystals (1.7A) of urate oxidase with three different inhibitors. The structure of these complexes gave us some new information about the urate oxidase enzymatic mechanism which is still unknown.1.N. Colloc'h *et al*, Nat. Struct. Biol. **4**, 11, 947-952 (1997). 2. D. Vivares & F. Bonnete, Acta Cryst. D **58**, 472-479 (2002). 3. D. Vivares *et al*, Eur. Phys. J. E **9**, 15-25 (2002).

## 7-12

**Importance of the solvent on the structural behaviour of the 23 residue long fusion peptide, fp23, of gp 41 from HIV-1**

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It is well known that envelopped viruses such as human immunodeficiency virus (HIV) infect their target cell by specific binding to the cell membrane followed by fusion of the viral membrane with cellular membranes. In the case of HIV, the N-terminal 23 residue long FP23 fusion peptide of the gp41 subunit of the gp160 glycoprotein is assumed to be responsible for the initial step of the fusion process. The FP23 sequence AVGIGALFLGFLGAA$G_{16}$STMGARS displays 16 first mostly hydrophobic residues while the 7 further residues are more polar. Due to its strong apolar character FP23 is unsoluble in common solvents, and numerous previous studies used DMSO (Dimethylsulfoxyde) to solubilize the peptide. In this work we investigate by PM-IRRAS spectroscopy and Brewster angle microscopy the role of the solvent in the adsorption ability, the FP23 structure and morphology at the air/water interface of a Langmuir trough. Several solvents were used : pure DMSO, mixed DMSO/Acetonitrile and TFA/water. It appears that mixed TFA/water solvent favors the FP23 adsorption to the interface compared to DMSO, but remains longer at the air/water interface. The DMSO favors an α-helix folding at low lateral pressure while TFA/water mixture favors highly organized β-sheets at the interface. Furthermore the morphology of the FP23 monolayers formed at the air/water interface are very different.whether the solvent used. This study emphazises that the choice of FP23 solvent may strongly influence the structural and morphological behaviour of the peptide. Consequently great care has to be taken for the interpretation of its mechanism of action.

## *Posters*

*- Protein structure dynamics and functions (II) -*

## 7-13

### Sequential unfolding of fibronectin via a four state mechanism

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Fibronectin (FN) is an extracellular matrix (ECM) protein, found soluble in corporal fluids or as an insoluble fibrillar component incorporated in the ECM. This phenomenon implicates structural changes that expose FN binding sites and activate the protein to promote intermolecular interactions with other FN. We have investigated, using fluorescence and circular dichroism spectroscopy, the unfolding process of human fibronectin induced by urea in different ionic strength conditions. Whatever the ionic strength conditions, the equilibrium unfolding data are well described by a four-state equilibrium model N<=>I1<=>I2<=>U. Fitting this model to experimental values, we have determined the free energy change for the different steps. We found that 10.4+/-3 kcal/mol are needed for the N<=>I1 transition. Such comparable values of free energy change are generally associated to a partial unfolding of type III domain. For the I1<=>I2 transition, we observed that the free energy change is 7.65+/-0.2 kcal/mol at low ionic strength condition but is twice less in high ionic strength conditions. This result is consistent with observations, which indicate that, from partially unfolded forms a complete unfolding of type III domain necessitates about 5kcal/mol. The third step, I2<=>U, represents the complete unfolding of fibronectin, with a free energy change of 14.6+/-4.2 kcal/mol in low ionic strength whereas this energy is again twice lower in high ionic strength condition. This hierarchical unfolding of fibronectin, as well as the stability of the different intermediate states controlled by ionic strength demonstrated here, could be important for the understanding of activation of matrix assembly.

## *Posters*

*- Single molecule biophysics -*

## 8-1

### Peptide coated semiconductor nanocrystals as probes for single molecule fluorescence microscopy

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Colloidal semiconductor nanocrystals (quantum dots) have recently been used as fluorescent probes for biological labeling. Their unique photophysical properties make them very attractive probes for cell imaging as well as for single molecule fluorescence spectroscopy. Cadmium selenide/zinc sulfide (CdSe/ZnS) nanocrystals are among the most common semiconductor particles studied. "Hot soap" synthesis of CdSe/ZnS yield high quality nanocrystals but requires hydrophobic surfactants such as Trioctyl Phosphine Oxide (TOPO) in order to control the growth of the nanoparticles. For biological imaging, a surface chemistry is then required to remove the surfactant molecules and solubilize the nanocrystals in aqueous solutions. Peptides were used as organic coat on the surface of CdSe/ZnS nanocrystals. The peptides provide aqueous buffers solubility and bio-compatibility to inorganic CdSe/ZnS nanoparticles by mean of surface recognition domains in the peptide sequences. The peptide coated nanocrystals are monodisperse, stable and have photophysical properties similar to that of TOPO coated particles. Modulation of the surface properties of the nanocrystals and direct bio-activation could be achieved using peptides of various amino acids sequences. Single molecule detection of peptide coated CdSe/ZnS was achieved by fluorescence confocal microscopy.

## 8-2

### Power spectrum analysis for optical tweezers: Precise calibration of forces

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The force exerted by an optical trap on a dielectric bead in a fluid is often found by fitting a Lorentzian to the power spectrum of the Brownian motion of the bead in the trap. We present explicit functions of the experimental power spectrum that give the values of the fitted parameters, including error bars and correlations, for the best such chi-squared-fit in a given frequency range. We use these functions to determine the information-content of various parts of the power spectrum, and find, at odds with lore, much information at relatively high frequencies. Applying the method to real data, we obtain perfect fits and calibrate tweezers with per-mil precision when the trapping force is not too strong. Relatively strong traps have power spectra that cannot be fitted properly with any Lorentzian, we find. A better understanding of the power spectrum than the Lorentzian provides, is then needed. We provide it by using new results for a popular photo-detection system, and old and new theory for Brownian motion in an incompressible fluid. We then calibrate tweezers and photo-detection system simultaneously in a manner that makes tweezers a tool of precision for force spectroscopy, local viscometry, and probably other applications

## 8-3

### G-quadruplex DNA structures studied by AFM

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G-quadruplex DNA are cyclic arrays of four hydrogen bond guanine (G) bases in which each base act as both donor and acceptor of two hydrogen bonds with other guanines, and the pairing between bases is Hoogsteen type. Self-assembled DNA networks have been shown for guanine rich oligonucleotides, where then exhibit semiconductor characteristics. Ours experiments were carried out to study the self-assembled formation dependence with G-quadruplex DNA concentration and ions concentration. Additionally, we used AFM images to elucidate specific guanine rich oligonucleotide structures under physiological conditions in order to use it as a potential target for therapeutic treatments. Once, these structures have been investigated due to their possible relevance to the recombinational events at the immunological effects.

## 8-4

### Force measurement on a transcribing T7 RNA polymerase

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We have performed single molecule experiments to measure the influence of a mechanical force on a transcribing T7 RNA polymerase (T7RNAP), as it translocates along a DNA template. This single unit motor enzyme is highly processive, exceptionally fast and structurally very related to Pol-I family including HIV 1 reverse transcriptase. The T7RNAP are prepared with a biotin tag and are attached onto a streptavidin-coated surface. A DNA construction has been prepared with a promoter sequence at one end and a digoxygenin ligand at the other end. The DNA is held at one end by the polymerase and the other end is attached to a microscopic bead covered with an antibody against digoxygenin. Using optical tweezers, we can capture the bead and measure the force that is developped during transcription. We present velocity measurements performed with an applied force in the range 5-20 pN, and at different nucleotide concentrations. We deduce from those measurements that the forward motion of the polymerase is correlated with nucleotide binding, and not directely to nucleotide hydrolysis.

## *Posters*

*- Single molecule biophysics -*

## 8-5

### Cell signalling investigated by single molecule spectroscopy

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Coordination of the interaction between the numerous cells of higher organisms relies upon complex signalling pathways. Receptors expressed on the cell surface trigger cascades of events upon binding of specific ligands. Single-molecule spectroscopy (SMS) and fluorescence correlation spectroscopy (FCS) allow the observation of the interplay between different ligands, receptors, and enzymes. Furthermore, the various conformations adopted by these receptors can be investigated by SMS, while conventional techniques probe only properties averaged over all possible conformations. We are focussing on two particular classes of receptors: (i) Upon binding of a ligand, G-protein coupled receptors activate G-proteins, which then regulate the activity of other enzymes and receptors triggering the signalling cascade. SMS and FCS will be used to investigate molecular interactions via properly labelled proteins. (ii) Signalling events during cytotoxic T-cell activation will be observed by SMS. Cytotoxic T-cell receptors bind to corresponding counter-receptors on target cells, the Major Histo-compatibility Complexes (MHC), which expose fragments of peptides. This allows the T-cell to recognize infected cells and to get activated. A description of these projects as well as recent experimental results will be presented.

## 8-6

### Physical properties of kinetin

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Kinetin belongs to a group of cytokinins, small molecules of a very important biological function. We shall present experimental data characterazing chemical and physical properties of kinetin, such as solubility, crystallization, molecular dynamics and phase transition in a single crystal will be described. In conclusion we shall discuss the relation between these properties and the biological activity of the kinetin molecule. The presentation includes data received in cooperation with students and scientists form Adam Mickiewicz University and Institutes of Polish Academy of Sciences in Poland

## 8-7

### Photoinduced hydration in pyridoxal 5'-Phosphate

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Pyridoxal 5'-phosphate (PLP) and related aldehydes have characteristic electronic absorption spectra that have been the subject of several previous investigations. Tautomeric and ionic states of the pyridoxal molecule has allowed the reactions of many PLP-dependent enzymes to be monitored by multiwavelength stopped-flow spectroscopy to obtain mechanistic information. In this work the photoinduced tautomerization and/or hydration of PLP have been studied in detail at 25 C and several pH by using a Biologic SFM-20 mixer coupled to a J&M Tidas16 256 diode array for multiwavelength data collection. The obtained spectra were analysed with lognormal curves which permitted the quantitative description of equilibria for hydration and/or tautomerization. The irradiation of a solution of pyridoxal 5'-phosphate by a 75W xenon lamp changes the ratio of the aldehyde-hydrates forms of PLP in aqueous solution. At low pH the hydrate form of PLP predominates over the aldehyde form and the radiation does not change the area of this band. At neutral pH the area of the aldehyde form of PLP decreases and the hydrate form increases its area with the same rate constant. At high pH the aldehyde form of the anionic PLP decreases its area and the hydrate form increases.

## 8-8

### Single molecule FRET studies of the human telomeric G-quadruplex structure and dynamics

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We have investigated the human telomeric intramolecular G-quadraplex using single molecule fluorescence resonance energy transfer. Studies of the conformational heterogeneity revealed two stable conformations in both sodium and potassium buffer with small differences between their enthalpy and entropy. Both conformations are opened by 21 base complementary DNA at the same rate, although this rate depends on the type of cation present. Temperature dependent studies in 100 mM KCl gave apparent activation energies and entropies of 6.4+-0.4 kcal/mol, and -52.3+-1.4 cal/(molK) indicating that structural changes in the quadruplex are entropically driven and can occur easily. In contrast, in sodium chloride the respective figures were 14.9+-0.2 kcal/mol and -23.0+-0.8 cal/(molK), suggesting a larger enthalpic barrier. Molecular modelling and CD measurements suggest that the two conformations might be the parallel and anti-parallel structures already found for intramolecular quadruplexes. The data is consistent with these two conformations interconverting, via a unfolded or partially folded state faster than the rate of hybridisation, on a time scale between 1 ms and 200 s. This work suggests that under physiological conditions both structures coexist and rapidly interconvert.

## *Posters*
*- Single molecule biophysics -*

## 8-9

### Ligand-binding and gating of single ion channels: combined fluorescende and electrophysiology experiments

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We have designed a set-up for simultaneous measurements of ligand-binding and gating of ion channels by combined single molecule spectroscopy and single channel electrophysiology. Ligand gated ion channels (LGIC) play essential roles in cellular signal transduction processes and are directly or indirectly targeted by many drugs. A typical LGIC is the nicotinic acetylcholine receptor (nAChR) that plays a key role in synaptic signalling and has been hence studied intensively, but detailed knowledge on channel function at a molecular level is still missing: Important questions are for example how ligands bind and how the channel opening is performed by structural changes in the protein. We use fluorescent agonists and antagonists in order to observe ligand binding to the receptor by single molecule fluorescence spectroscopy. Simultaneous patch clamp experiments give evidence on the ion channel activity of the receptor. The nAChR can adopt at least four discrete states corresponding to ligand binding and opening of the channel. Working on a single molecule level allows the direct observation of fluctuations between different conformational states of the receptor. Results of first experiments will be presented.

## 8-10

### Properties of the bacteriophage Phi29 capsid investigated with Scanning Force Microscopy

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Viruses with their relatively simple well-studied structure are the ideal candidates for a physical microscopic investigation which can give insight into the design of this amazing molecular machines. The object of our investigation is bacteriophage Phi29 which infects Bacillus subtilis. The structure of the bacteriophage has been determined in different stages of its maturation using cryo-electron microscopy. The virus itself is quite remarkable in structure and capabilities. The 42x54nm capsid has to accommodate a 20 kbp piece of DNA. In order to fit the DNA inside the shell, the DNA has to be compacted to near crystalline densities. Recent single molecule experiments of the packaging of DNA into the f29 capsid provided an estimation of the tensile strength of the shell of at least 100 MPa. This is roughly the tensile strength of a typical aluminum alloy. Using SFM in so-called jumping mode we imaged the proheads applying different forces in order to see their deformability. We observe terraces in the topography, possibly due to the nonhomogeneous protein structure of the shell. Then pushing on empty prohead and recording the force-distance curves we detect its elastic response at different loads till we start to destroy it. This might give us an indication of its tensile strength. Finally, comparing the behavior of empty prohead with full ones in response to outside pressure induced from the SFM tip, we can obtain the pressure which the DNA applies to the inside of the virus.

## 8-11

### Rearrangement Dynamics of a Single Chromatin Fiber studied by Magnetic Tweezers

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Chromatin is a universal template for eukaryotic DNA that plays a key role in genomic functions. The complex structure of chromatin consists of wrapping DNA, in left handed superhelical turns, around histone octamers to form nucleosomes. These nucleosomes are spaced along the DNA molecule and interact to form higher-order structures. The dynamics of this structure have long remained hard to monitor with traditional molecular biology techniques. Single Molecule experiments seem to be a powerful tool to approach real time chromatin dynamics. We propose here a magnetic tweezers set-up which follows chromatin assembly and gains insights into chromatin strucure. Magnetic tweezers allow fine tuning of two key parameters: (i) force, in the range of 0,05 to 20 pN; and (ii) torsion, by monitoring the number of turns of the DNA molecule. We study chromatin assembly and rearrangement dynamics in cell free systems derived mainly from Xenopus eggs. We will present our latest findings on this topic

## 8-12

### Real time fluorescence imaging of DNA ejection from a single phage particle

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The mechanism by which phages transfer their genome through bacterial membranes is badly understood. The coliphage T5 offers the unique opportunity to study this mechanism in vitro since its interaction with its purified membrane receptor, FhuA, is sufficient to trigger the release of the DNA (121 kbp) either in solution or in liposomes (Lambert et al. (2000), Proc. Natl.Acad. Sci., 97, 7248; Bohm et al. (2001) Current Biology 11, 1168). Here, we report confocal fluorescence experiments that allow real time visualization of DNA ejection from a single phage particle. Phages T5 were adsorbed onto the surface of a microfluidic cell. FhuA was then injected into the cell causing the ejection of the DNA. Release of the DNA of a single phage was observed using a fluorescent DNA intercalant. DNA ejection occurred into two well-defined steps as observed in vivo: about 8% of the DNA was first released; It remained attached to the phage. the rest of the DNA was then released. The DNA still attached to the virus could be mechanically stretched back and forth. DNA ejection took place within a few hundred msec. These experiments allow for the first time to visualize the process of DNA ejection and to analyze the dynamic of the process on single viral particles. This system should permit to measure the forces that retain the DNA into the capsid and allow it to be transferred into the host upon infection.

## *Posters*

*- Single molecule biophysics -*

## 8-13

### Investigating the stepping mechanism of molecular motor myosin V

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The stepping kinetics of molecular motor Myosin V is investigated using single molecule fluorescent microscopy. While moving along an actin filament, the two-headed motor performs 36 nm steps, but the model for the actual stepping mechanism, whether it moves in a head-over-head or inch-worm-like manner, has not yet been confirmed. For this purpose, calmoduline in the vicinity of one of the catalytic domains of the molecule was exchanged by a fluorescently labelled one, which enabled us observations of single motor domain movement while stepping along an actin filament. Single steps were observed and step length was found to be ~ 72 nm, which clearly supports the head-over-head model for myosin movement. Comparison between weakly labelled, where only one calmoduline was exchanged, and strongly labelled myosin molecules was made and the results are consistent with the proposed model.

## 8-14

### Holliday junction studied by Single Molecule Micromanipulation

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Homologous Recombination or Strand Exchange Repair Pathways are essential mechanisms ensuring genetic diversity and conservation of genetic information. An important intermediate of these processes is the Holliday junction: a four branched DNA structure which allow single strand exchange between two dsDNA molecules. Exchange of single strands occurs during the Holliday junction migration process. We study mechanical properties of the Holliday junction using Magnetic Tweezers. We can stretch and supercoil a single DNA molecule attached by its ends between the sample surface and a magnetic micro-bead. We use a palindromic DNA molecule made by head to head ligation of the same two fragments. When the molecule is negatively supercoiled, we can have the formation of a Holliday junction. One can then induce junction migration by winding/unwinding the molecule. We study this migration process under the effect of supercoiling, force and ionic conditions.

## 8-15

### Sequence-specific fluorescent labeling of double-stranded DNA observed at the single molecule level

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We achieved fluorescent labeling of a short sequence of double-stranded DNA (dsDNA) by ligating a labeled dsDNA fragment to a stem-loop triplex forming oligonucleotide (TFO). The TFO first winds around the target dsDNA sequence by ligand-induced triple helix formation, then its extremities hybridize to each other, leaving a dangling single-stranded sequence, which is finally ligated to a fluorescent double-stranded DNA fragment using T4 DNA ligase. As a target we chose a non repeted 15 bp oligopurine-oligopyrimidine sequence on lambda DNA. Lambda DNA was labeled, combed on a microscope slide by a receding meniscus and then visualized by fluorescence microscopy. The label was found to be attached at a specific position located at 4.2 +/- 0.5 kbp from one end of the molecule, in agreement with the location of the target sequence for triple helix formation (4.4 kpb from the end). Besides, an alternative combing process was noticed in which a DNA molecule gets attached to the combing slide from the label rather than from one of its ends. The method described herein provides a versatile tool for the detection of very short sequences of dsDNA and offers new perspectives in the micromanipulation of single DNA molecules.

## 8-16

### Single molecule observations of green fluorescent protein unfolding in solutions and gels

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Unfolding of GFP was investigated in solution and in silica gels both on concentrated samples and at single molecule level under the action of the denaturant GuHCl. In solution, at 37C, the midpoint falls at 2.5 M, close to the results found in the gel. The unfolding can be described by a two-state process with rate constants of 0.20 and 0.006 min-1, in solution, and 0.38 and 0.0018 min-1, in silica gels, at 3 M GuHCl. When monitoring the unfolding process in silica gels (10-20 molecules of GFP/image) by confocal microscopy in the absence of denaturant, the number of fluorescent molecules does not change appreciably for at least 16 hours at image acquisition rate of 1/ 900 s, whereas, at higher image acquisition rates (1/0.5 s), significant bleaching of GFP-mut2 is observed. In the presence of denaturant the number of fluorescent molecules falls at rates consistent with those determined in concentrated protein gels. Summarizing, the dynamic properties of the mutant GFP-mut2, encapsulated in wet silica gels are found to be close to those of the protein in solution. Furthermore, as a test of protein renaturation, it is found that when the gels containing denatured GFP-mut2 are soaked in denaturant-free buffers, most of the GFP molecules revert to their previous fluorescence level.

## *Posters*

*- Single molecule biophysics -*

## 8-17

**Fluorescence fluctuation spectroscopy and photon counting histogram analysis for investigation fo ligand binding to G-protein coupled receptors**

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G-Protein Coupled Receptors (GPCRs) are integral membrane proteins involved in a wide range of signaling pathways, making them drug targets for a variety of diseases. Possible ligands are proteins, peptides, amino acids, fatty acid derivatives, monoamines, purines. For binding measurements we used GPCRs in lipid vesicles and a fluorescent labeled peptide ligand (TMR-PL) as a model system. The fluorescence fluctuation signal from a sensitive volume of about 1 fl was measured using a confocal microscope. The fluorescence of TMR-PL upon binding to GPCR does not change. The slower diffusion of the vesicles and the high number of receptors on the vesicles allow us to distinguish between free and bound TMR-PL in the experiments. Photon Counting Histogram (PCH) analysis is sensitive to the different particle brightness of free ligand and vesicles with bound ligands. Autocorrelation functions of the fluorescent signal reveal the different diffusion times of free TMR-PL and TMR-PL bound to lipid vesicles. Our results demonstrate that fluorescence fluctuation spectroscopy, and particularly the PCH analysis, is a powerful method in homogeneous membrane receptor binding assays.

## 8-18

**Single molecule imaging using fluorophores and quantum dots reveals multiple diffusive states of glycine receptors**

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Analyzing the dynamics of receptors at the neuronal surface is essential to understand the organization of the postsynaptic membrane and its modifications occurring during synaptogenesis or synaptic plasticity. To address this issue, we use single-particle tracking of endogeneous glycine receptor (GlyR) in cultures of spinal cord neurons and followed the receptor motion with primary antibody (mab2b) coupled to small probes allowing access to intrasynaptic compartments. Using Cy3-mab2b, we characterized rapid extrasynaptic motion, with a diffusion coefficient D»10^(-1) mm2/s. At synapses, receptors diffuse slower (D»10^(-2)mm2/s) in confined spaces (about 200 nm). However, fast photobleaching of the dyes (» 5 s) considerably limit the acquisition time. To overcome this problem, we imaged the receptors using semiconductor quantum dots (QDs) which enabled us to visualize trajectories for several minutes and to detect multiple intersynaptic exchanges in which GlyR alternate between the fast and confined diffusive states described above. For the synaptic GlyRs, we compared their motion when imaged immediately after labeling and two hours later. In the latter case, preliminary results indicate a decrease in diffusion coefficient by about a factor 5. In addition, electron microscopy unambiguously showed that QD-labeled GlyR, mostly perisynaptic after labeling, are increasingly intrasynaptic as function of time. Altogether, these results: (i) directly prove that GlyR can enter synapses by a diffusion-trap mechanism where it is stabilized, (ii) validate the use of QDs for single molecule tracking, (iii) suggest the existence of two states for synaptic receptors with perisynaptic receptors more loosely anchored to the scaffold.

## 8-19

**Multi-Path Total Internal Reflection Microscopy (MP-TIR) for single molecule analysis of transcription complexes**

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We have developed a new objective-based Total-Internal-Reflection Fluorescence microscope that allows selective excitation of single molecules with light of two wavelengths ("colors") or three polarizations. The microscope is used for single-molecule Forster Resonance Energy Transfer (smFRET) or fluorescence polarization (smFP) studies of transcription initiation and elongation by Escherichia coli RNA polymerase. Two different paths for the incoming light (parallel or perpendicular to the sample plane) are used, allowing us to focus the laser beams off-axis at the back focal plane of a high numerical aperture objective, at two different positions. In the two-color smFRET mode, alternation between a 514-nm and a 638-nm laser (ALEX-TIR) allows selective excitation of donor and acceptor fluorophores, and measurement of donor and acceptor emission. The advantage of ALEX-TIR over single-laser-excitation TIR is the precise determination of the FRET efficiency using the sensitized emission of the acceptor method, as well as separation of low- or zero-FRET species from donor-only species. In the 3-polarization mode, the 514-nm excitation alternates between s and p linear polarizations in one path, and p in the second path. The polarization of the evanescent field is thus selectively polarized in each dimension, allowing determination of the absolute orientation of fluorescent dipoles. Results obtained using the MP-TIR method on DNA model systems and on RNA-polymerase transcription complexes will be presented.

## 8-20

**Single molecule near field microscopy in cell biology: A quantitative protein study on the cell membrane**

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Organization of membrane components and cell functioning are two closely related subjects. Understanding one reveals the other. Near-field Optical Microscopy (NSOM) in combination with single molecule sensitivity is a powerful technique to investigate densely packed molecular components in a quantitative way. As a high resolution surface sensitive scanning probe technique NSOM provides an optical resolution of about 50nm, is able to discriminate single molecules on the cell membrane of fully intact cells and maps the membrane topography. Here we will discuss the application of NSOM to investigate the molecular organization of proteins on different types of cells such as Dendritic- and T-cells. In the case of Dendritic cells (DCs) we examined protein domains that could be characterized in terms of the number of domain components (ranging from a few to more than 100), inter-domain (typical 200nm) and also intra-domain distances (about 10nm). We will also report on the co-localization of different protein types on the membrane of T-cells. These studies help to understand the specific cell function in different cellular processes

## *Posters*

*- Single molecule biophysics -*

## 8-21

### Photobleaching and compartimentation : In between FCS and FRAP

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The photobleaching phenomenon is the crucial process that allows, in FRAP and similar techniques, to estimate the diffusion constant from the time evolution of the fluorescence intensity. On the contrary, in FCS, photobleaching is generally considered as an undesirable event that one tries to get rid of. However, there is conceptually no reason to make any distinction between FCS and FRAP when photobleaching occurs. These two techniques probe the dynamical behaviour of the fluorochromes on different time scales : while FRAP gives access to relatively long time scale ($\geq$ ms), FCS scans intensity data from less than 1 µs to tens of s. Our goal is to bridge the gap between these two approaches and to demonstrate that photobleaching is not a drawback for FCS, but that it does give information about diffusion and compartimentation.
Since living cells are very complex, unstable and fragile media it appears relevant, as a first step, to perform experiments with controllable bio-mimetic objects, such as giant unilamellar vesicles (GUV), from 10 to 100 µm in diameter. Successful experiments have been performed with FITC and Lucifer Yellow CH (LY-CH) dyes, known as being easily bleachable fluorophores. Using the characteristic length of compartimentation, we related the intensity fluctuations on the time scale of a few hundreds of µs (as seen from FCS) to the overall, comparatively slow, decay of the fluorescence intensity. Experimental results are compared with theoretical models and numerical simulations

## 8-22

### Studying torsional properties of biological macromolecules by optical tweezers

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Characterization of torsional properties is often crucial in understanding the function of biological macromolecules. Here we introduce a novel manipulation method by which it is possible to apply and measure torque on microscopic objects. In this procedure the applied torque can be turned on and off, it is controlled independently of the grabbing force of the tweezers during the manipulation process.
The method is based on the observation that flat objects are oriented in an optical trap formed by linearly polarized light. The orienting torque originates from the anisotropic scattering of polarized light by the trapped particle. The phenomenon is characterized in detail, the physics is analysed. Microscopic particles are produced by photopolymerisation that exploit this orientation effect. A tool is developed to manuipulate biological macromolecules.
The power of the method is demonstrated. Single actin filaments and DNA molecules are pulled and twisted by laser tweezers. The pulling and twisting forces are adjusted and measured independently. The flexibility of the method gives new insight into the mechanics of biological macromolecules.

## 8-23

### Visualization of vesicle fusion on a biochip - towards structural and functional studies on a single protein

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This work is focused in the development of methods for simultaneous electrical and optical measurements of membrane proteins at the single molecule level. Peptides such as alamethicin and gramicidin are incorporated into planar lipid membranes formed on an aperture (10 - 100 µm in diameter) of micro-fabricated silicon chips. Vesicle fusion into these membranes is monitored by spreading of fluorescence of labelled proteoliposomes and by changes of electrical current due to the activity of channels. Reconstitution of membrane proteins will be the next step. Special attention will be given on the incorporation and immobilization of ATP-synthase. Polystyrene beads attached to a specific portion of ATP-synthase will allow the application of optical tweezers to assist its rotation leading to an extended characterisation of the mechanism of synthesis of ATP. For single molecule visualization it is necessary an appropriate micro-fluidic system due to the short working distance of near-field optical techniques. To solve this technical problem and maintain a water-like environment next to the membrane a thin layer of agar gel is used. We hope to produce a stabilizing cushion for the lipid membrane and open the possibility for anchoring membrane proteins to avoid bi-dimensional diffusion