Evolutionary Methods for Studying the Structure and Function of Proteins

S09-01

Structure-Based Analysis of Lysozyme Evolution

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The lysozymes from chicken egg-white (HEWL), Embden goose (GEWL) and bacteriophage T4 (T4L) have essentially unrelated amino acid sequences but have similarities in their three-dimensional structures that suggests that they evolved from a common precursor.

Cleavage of saccharide substrates by HEWL yields the β-anomer whereas hydrolysis by T4L gives the α-anomer. Replacement of Thr 26 in the active site of T4 lysozyme by Glu gives a mutant enzyme (T26E) that covalently traps the product of hydrolysis in the active site. In contrast, the mutant T26H is enzymatically active, but in distinction to wild-type gives the β-anomer. Thus, in this instance the mechanism of action of the enzyme can be altered by a single amino acid replacement. It suggests that the retention of similar catalytic activity need not be a prerequisite for divergent evolution.

Lysozyme evolution is being further explored by the construction of "chimeric" lysozymes in which segments of P22 lysozyme are substituted into T4 lysozyme.

S09-02

Evolving Smaller Protein Binding Domains. James A. Wells, Brian C. Cunningham, Betty Li, and Andrew Braisted, Department of Protein Engineering, Genentech, Inc., South San Francisco, CA 94080.

Protein domains are generally considered to be indivisible elements of protein structure and act as portable modules which afford great functional diversity. We were interested in testing whether smaller protein domains could be constructed that maintain function.

Atrial Natiuretic Petide (ANP) and the B-domain of Protein A are polypeptides of 28 and 59 residues, respectively, which bind their cognate receptor using discontinuous determinants. Attempts to make simple deletions in these peptides and maintain binding affinity have failed. Phage display can be a powerful tool for peptide and protein design when used in conjunction with high resolution structural information. Using an interative process of rational protein design and phage methods to rectify defects inherent in the design we have been able to reduce by half the size of ANP and the IgG binding domain of protein A. Such an approach may facilitate the design of small peptidometics from larger peptides or proteins.

S09-03

LINKING PEPTIDE STRUCTURE AND FUNCTION TO FILAMENTOUS BACTERIOPHAGE AMPLIFICATION.
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Large collections of peptides can be synthesised in vivo by fusing their coding sequences to a variety of bacterial genes. If the fusion partner is a bacteriophage coat gene the peptides will be eventually displayed on the surface of bacteriophage particles available for interaction with other molecules. Phage suspensions of approximately 10exp13, displaying as many as 10exp8 different peptides, can then be searched by affinity methods for those elements that bind a given target of interest. The bound phage is then amplified, thus linking bacteriophage amplification to the structural properties of the displayed peptide.
Subtle variations of this standard protocol have permitted to extend the applications of phage display technology to the study of protein modifications, enzyme mechanisms and protein folding.

S09-04

DNA SEQUENCE EVOLUTION BY SEXUAL PCR.
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Existing DNA mutagenesis methods, such as oligonucleotide-directed mutagenesis, error-prone PCR and mutator strains have important limitations when applied recursively for multiple cycles. We have developed a simple method for homologous recombination of pools of related genes *in vitro*, called DNA shuffling, or sexual PCR. Libraries of chimeric genes can be constructed from different species or from related genes. Using five cycles of mutagenic DNA shuffling, followed by back-crossing, we have increased the resistance of *E. coli* expressing ß-lactamase to the antibiotic cefotaxime by 32,000 fold, whereas cassette mutagenesis and error-prone PCR yielded only a 16 fold increase. We have applied shuffling for the directed evolution of human antibodies. Using a circular shuffling format coupled with selection we can perform 'molecular breeding' of large plasmids encoding whole operons or enzymatic pathways, as well as whole viruses. This approach is simple, does not require any sequence information, and is compatible with many host cell types.

S09-05

Docking in a flexible binding site.
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A novel method for docking ligands in a flexible binding site is presented. The method consists of the following steps. First the ligand is seeded randomly in the binding site. The complex is minimized with a modified force field which avoids stability problems during minimization. Then the minima are evaluated according to their approximated free energy and shielded electrostatic interaction, which are calculated with a continuum approximation. In the last step the best minima in terms of free energy are subjected to a Monte-Carlo minimization with a Metropolis criterion which takes solvation into account.
The method has been successfully applied to two different systems. The first one is the binding of progesterone and 5-β-androstane-3-17-dione to the Fab fragment of a steroid binding antibody (DB3). A comparison of the crystal structures of the free and the complexed form, reveals that attempts to model binding must take protein rearrangements into account. Furthermore, different ligands bind in two different orientations which poses an additional challenge to docking algorithms. The second test case is the docking of NAPAP to human α-thrombin. In contrast to steroids NAPAP is a very flexible ligand.
The efficiency and limitations of the algorithm are demonstrated on these two proteins. In the steroid binding study the best minima in terms of free energy lie within 1.2Å RMS deviation from the crystal structure of the corresponding complexes, while most of NAPAP was docked in a conformation almost identical to the crystal structure of the complex. Applications in the prediction of transition state binding, and possibilities for further development of the method are suggested.

S09-06

REAL-TIME INTERACTIVE VISUALIZATION OF THE ACTIVE SITE OF ASPARTATE AMINOTRANSFERASE
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The computer-based visualization of molecular models has become an indispensable tool in the study of biological macromolecules. However, programs that allow the interactive exploration of macromolecules in a real-time fly-through mode have so far been lacking due to the enormous amount of data that have to be visualized. Using high-performance computer graphics workstations and software we have succeeded in creating virtual macromolecular environments for the interactive visualization and exploration of proteins . The application allows the use of different representation models together with stereo view, sound effects and text information. As an example, a model of aspartate aminotransferase, a pyridoxal-5`-phosphate dependent enzyme, has been displayed. The application should prove useful in undergraduate instruction and for introducing laypersons to the world of macromolecular structure and interaction.

S09-07

SWISS-PDBVIEWER:

A NEW FAST AND EASY TO USE PDB VIEWER FOR THE MACINTOSH.

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Swiss-PdbViewer is a Macintosh application that can display PDB files. Several proteins can be analyzed at the same time and can be piled-up in 3D. RMS can then be calculated on selected amino acids of the aligned proteins, taking into account some or all atoms; allowing easy comparison of relevant parts. Swiss-PdbViewer can also measure angles, distances and torsions with simple mouse clicks, thanks to the intuitive graphic interface. A control Panel regroups display facilities so that atom types and names, sidechain display/undisplay and amino acid colors are accessible with a single click. H-bonds can be estimated for the whole molecule even if the PDB file does not provide explicit H atoms, and it is possible to mutate amino acids sidechains by browsing a rotamer library. During the mutation process, H-bonds and steric hindrances are automatically estimated and displayed in real time, facilitating the best rotamer choice.
The program is under constant improvement and can be retrieved from ExPASy [1]. The short-term purpose is to develop a multi-platform modeling package that will allow multiple alignements, and direct submission of modeling requests to the Swiss-Model server [2].

[1] http://expasy.hcuge.ch/swissmod/Swiss-PdbViewer/mainpage.html
[2] http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html

S09-08

CONSTRUCTION AND CHARACTERIZATION OF AN ANTI-BODY SCF$_v$ FRAGMENT LACKING THE DISULFIDE BOND IN THE HEAVY CHAIN

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Disulfide bonds have been very well conserved during evolution within all proteins of the immunoglobuline superfamily and are believed to serve an important function in stabilizing the ß-barrel that makes up the immunoglobuline fold. Still, some sequences of antibody V_H and V_L genes are known which are lacking the disulfide bond. We wished to investigate whether such sequences could be useful as a starting point for construction of disulfide bond free antibody fragments or for frameworks with improved thermodynamic stability. We have chosen the sequence of the antibody ABPC48 which is lacking the disulfide bond in V_H because of a C98Y mutation. Different scFv fragments (concerning arrangement of the V_H and V_L genes, and linker lengths) of the wt sequence and a Y98C mutant, which restores the missing disulfide bond, were constructed and expressed in *E. coli*. Functional, monomeric scFv protein in the orientation V_H-linker-V_L of the wt and mutant sequences could be purified using antigen affinity chromatography, and the stability of the proteins was measured by equilibrium urea and guanidinium denaturation.

S09-09

Changing an Ampicillin - binding Antibody into a Beta-Lactamase by Protein Engineering

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A library of antibody single-chain Fv fragments derived from mice immunized against the beta-lactam antibiotic ampicillin was screened by phage-display. Clones with marked selectivity for intact ampicillin versus hydrolyzed ampicillin were sequenced and found to be closely related. The putative structure of these scFv fragments was predicted by homology modelling, using closely related immunoglobulin structures as templates (VL: PDB entry 1nca, 85% sequence identity, VH: PDB entry 1jhl, 92% sequence identity). Ampicillin was docked to the model using a Monte-Carlo docking algorithm (MCSS), which allows for flexibility in ligand and antibody side chains, while the main chain structure was kept rigid. Model complexes showing reasonable interaction energies were compared to the results of epitope mapping studies with a series of ampicillin analogs. Based on those models, the possibility of confering catalytic activity to the antibody was investigated. The engineering of an extensive hydrogen-bonding network involved in the mechanism of type A and C (serine) beta-lactamases is unlikely to succeed, while the introduction of a catalytic zinc binding site to mimic the mechanism of type B (metallo-) beta-lactamases may be feasible. Once even weak catalytic activity is obtained, such antibodies would confer a selective advantage to bacteria grown in the presence of ampicillin and serve as a staring point to further improve catalytic activity, folding efficiency and stability of the antibody through evolutionary strategies.

S09-10

COSELECTION OF COGNATE ANTIBODY-ANTIGEN PAIRS BY SELECTIVELY-INFECTIVE PHAGES

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We have produced an antibody-displaying phage which is itself non-infectious and acquires the ability to infect cells only upon the occurrence of a cognate interaction between antibody and antigen. This selective infectivity is achieved by exploiting the modular structure of gIIIp. The first two domains (N1-N2), which have been shown to be responsible for the docking of the phage to the F-pili of male *E. coli* and subsequent penetration of the bacterial membrane, are linked to the antigen. As the partner for the binding reaction, the phage genome encodes a fusion of single-chain antibody to the C-terminal domain of gIIIp. We have developed a chloramphenicol resistant derivative of fd phage which encodes both antibody and antigen, and with which cognate pairs of antibodies and antigens can be selected. This system has a very low background and will allow simultaneous randomisation of antibody and antigen.

S09-11

EVOLUTIONARY IMPROVEMENT OF CATALYTIC ANTIBODIES BY METABOLIC SELECTION

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While tight binding of a ligand can be achieved in a multitude of ways, catalysis has far stricter geometric requirements, as there are few key interactions which have to be optimal. Antibodies have been shown to be catalytic for a multitude of reactions, yet most turnover rates have been rather modest. To solve this problem, we are attempting to harvest the power of directed evolution with a model system of antibodies with β-lactamase activity expressed in *Escherichia coli*.
Recently, libraries derived from mice immunized against ampicillin were successfully constructed which are now investigated by direct plating of antibody producing bacteria on ampicillin containing culture medium in order to find efficient catalysts. Simultaneously, the antibody library has been cloned in an improved phage display vector and was panned for identifying binding molecules which were then further on characterized by epitope mapping and computer modeling. The most interesting one, called aL2, will be mutagenized and screened for catalysis by plating on ampicillin.
Such 'selectable' antibodies are the ideal starting molecules for the use of evolutionary strategies in order to improve general antibody framework properties such as folding, stability and expression as well as *E. coli* strains suitable for efficient expression of recombinant antibody fragments.

S09-12

THE INNER BETA-SHEET OF THE V_L DOMAIN ACTS AS A FOLDING NUCLEUS FOR THE FOLDING OF THE SCFV FRAGMENT OF AN ANTIBODY

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The folding kinetics of the scFv fragment of the phosphorylcholine-binding antibody McPC603 were investigated by the use of fluorescence spectroscopy, nuclear magnetic resonance and mass spectrometry. All three methods gave evidence for the occurrence of a major kinetic intermediate during the refolding of the denatured, oxidized scFv fragment. This intermediate is formed within the first 30 seconds of folding and comprises exchange-protected amide protons of hydrophobic and aromatic amino acids, most of which are localized within the inner β-sheet of the V_L domain. Some of these amino acids are extremely conserved among different antibodies, suggesting the potential to form a kinetic intermediate in the folding reaction to be a conserved feature during the evolution of antibody variable domains.

S09-13

MODELING THE LIGAND BINDING SITE OF AN ANTI-DDT ANTIBODY

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We have designed a 24-residue peptide that shows strong binding of the insecticide DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl)ethane) (1). The postulated β-sheet conformation of this peptide was confirmed by CD spectroscopy. In order to be able to compare the structures of the ligand binding site of an artificial and a natural DDT-binding protein, several monoclonal anti-DDT antibodies were prepared (2) and their V_L and V_H domains sequenced using phage display technology. Based on strong sequence similarity with JE142 and 36-71, two monoclonal antibodies of known x-ray structure, a model of the variable domains of one of the anti-DDT antibodies was obtained using energy minimization and molecular dynamics calculations. Manual docking of DDT followed by additional molecular dynamics calculations revealed a potential binding site in which the CCl_3 and the two chlorophenyl groups of DDT were largely buried.

1. Moser, R., Thomas, R.M., Gutte, B. (1983) FEBS Lett. 157, 247.
2. Bürgisser, D., Frey, S., Gutte, B., Klauser, S. (1990) Biochem. Biophys. Res. Comm. 166, 1228.

S09-14

NMR solution structure of human cyclophilin A

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Cyclophilin A is an enzyme with peptidyl–prolyl *cis–trans* isomerase activity, which also functions as an intracellular receptor of cyclosporin A. Because of its high affinity for this immunosuppressive drug it plays an important role in the prevention of organ transplant rejection.

In this communication we present the NMR solution structure of the free, uncomplexed protein. Complete ^1H, ^{13}C, and ^{15}N resonance assignments have been obtained using heteronuclear NMR techniques. On this basis, 4101 distance constraints derived from nuclear Overhauser enhancements were collected as input for the calculation of the three-dimensional structure in solution: the average RMSD value relative to the mean coordinates is 0.5 Å for the backbone, and 0.9 Å for all heavy atoms of residues 2–165 of the 20 best DIANA conformers. The dynamic behavior of cyclophilin was also investigated using ^{15}N T_1, T_2 and $T_{1\rho}$ relaxation measurements. Special interest of this project is focussed on comparative studies with the cyclophilin–cyclosporin A complex, which may provide novel insights into the structural basis of the specific interactions of this complex with calcineurin.

S09-15

NMR solution structures of the DNA-binding domain residues 1–63 of the wild type 434 repressor and its [R10M] mutant.

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Two NMR structures of the DNA–binding domain of the 434 repressor, 434(1–63), have been determined in aqueous solution, one of the wild type sequence, and one of the mutant 434[R10M](1–63). The mutation removes a buried salt bridge between the side chains of Arg 10 and Glu 35. In addition to the standard structure determination, transient hydrogen bonds on the surface of the proteins were identified by pH titration experiments. A comparison of the two structures shows that their molecular architectures in solution are similar and include five α–helices extending from residues 2–13, 17–24, 28–35, 45–52 and 56–61. Significant differences between the structures of the wild type and mutant proteins are localized to the first three helices and include residues involved in DNA recognition. The [R10M] mutation of the 434 repressor leads to a slightly different orientation of the DNA-binding helix–turn–helix motif (residues 17–35) when compared with the wild type protein. A molecular mechanism for the structural rearrangement in the mutant is proposed.

S09-16

The NMR Solution Structure of the Pathogenesis-Related Protein P14A

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Nuclear magnetic resonance (NMR) spectroscopy was used to determine the three-dimensional structure of the 15 kDa pathogenesis-related protein P14A, which is induced in tomato plants as a response to pathogen infection. Due to its strong antifungal activity, P14A is of interest with regard to the generation of transgenic plants with improved host defense properties. The molecular mechanism of the antifungal activity of P14A is unknown so that the three-dimensional structure now has a central role as a lead for continued investigations on the mode of action.

The NMR structure of P14A was determined using both $^{15}N/^{13}C$ doubly-labelled and unlabelled protein. A set of about 2000 conformational constraints was collected, which resulted in a high-quality solution structure: the average RMSD value relative to the mean structure is 0.9Å for the backbone, and 1.3Å for all heavy atoms of the 20 best DIANA conformers. P14A contains four helices (I to IV) comprising residues 4–17, 27–40, 64–73 and 93–98, and a four-stranded β-sheet containing residues 24–25, 53–58, 104–111 and 117–124. These regular secondary structure elements form a complex α/β topology in which the helices I, III and IV are located above and the helix II below the plane defined by the β-sheet.

S09-17

The NMR structure of DnaJ(2–108) comprising the J-domain and the Gly/Phe-rich region of the chaperone DnaJ from *Escherichia Coli*.

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The NMR solution structure of the N-terminal 108-amino acid polypeptide of DnaJ from *Escherichia coli* is presented. The structure of the J-domain with residues 2–76 is well defined and contains four helices comprising residues 6–10, 18–32, 41–57, and 61–68. The helices II and III form an antiparallel helical coiled-coil, and the helices I and IV are both oriented approximately perpendicular to this arrangement. This topology leads to formation of a hydrophobic core involving side chains of all four helices. A clear-cut correlation is observed between the extent of conservation of hydrophobic residues in J-domains and their structural role in the cluster architecture. Furthermore, it is found that residues which have key roles for the specificity of the interaction of DnaJ-like proteins with their corresponding Hsp 70 counterparts, are located on the outer surfaces of the helices II and III, and in the loop connecting them. In contrast to the J-domain, the Gly/Phe-rich region with residues 77–108 is flexibly disordered in the NMR solution structure. However, additional studies of internal mobility based on measurements of ^{15}N spin relaxation times, heteronuclear ^{15}N-{^1H} NOEs and backbone amide proton exchange rates also show that the polypeptide segment comprising residues 90 to 101 does not have the properties of an ideal "random coil" but transiently adopts non-random conformations, which may play a crucial role for the stability of the ternary DnaJ–DnaK–polypeptide complex.

S09-18

Nuclear magnetic resonance studies of the hydration of the SH3 domain of human p^{56}lck in aqueous solution

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Solvation with water molecules is an important aspect of protein and DNA structure. In particular, hydration is of direct relevancy for the understanding of protein-ligand interactions, since the hydration waters may contribute to the molecular topology recognized by the ligand. Through observation of nuclear Overhauser effects (NOE), nuclear magnetic resonance (NMR) spectroscopy can be used to identify distinct hydration sites in proteins and nucleic acids in aqueous solution, and to measure the lifetimes of hydration water molecules in these hydration sites (G. Otting, E. Liepinsh and K. Wüthrich, *Science* 254, 974-980 (1991)). Here we report NMR studies of the hydration of the SH3 domain of human p^{56}lck. In the full-length SH3, quite uniform distribution of hydroxyl protons of serines and threonines over the entire protein surface makes unique assignments for the detected protein-water NOEs difficult. We have therefore truncated the native protein at both chain ends to remove hydroxyl-carrying amino acids from one side of the folded protein, thus enabling to resolve certain assignment ambiguities. Based on these experiments, a preliminary picture of the hydration of the SH3 domain will be presented. These investigations also form the basis for studies of SH3 complexation with proline-rich ligands in aqueous solution.

S09-19

FAMILY PROFILE ANALYSIS, A NEW ALGORITHM FOR DETECTING EVOLUTIONARY RELATIONSHIPS AMONG PROTEIN FAMILIES.

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In the face of the ever increasing sequence information (to date 43,000 amino acid sequences from 8,000 superfamilies), sensitive, automated algorithms assessing evolutionary relationships among proteins have become a growing need. Profile analysis which compares a set of homologous probe sequences with the target sequence, is currently the most sensitive method available for this purpose. We have developed a new algorithm which compares the probe family with all available sequences of the target family. The increased input of sequence information on the part of the target significantly expands the range in which structural relationships can be quantitatively verified. The new algorithm proved successful in a number of cases in which the evolutionary relationships as yet could only be detected by comparison of 3-D structures.

S09-20

MOLECULAR EVOLUTION OF PYRIDOXAL-5'-PHOSPHATE-DEPENDENT ENZYMES

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Application of a newly developed algorithm (Family Profile Analysis) showed the existence of the following evolutionary lineages of pyridoxal-5'-phosphate(PLP)-dependent enzymes: alpha/gamma family (mostly catalysing covalency changes at C alpha or C alpha + beta + gamma, respectively), beta family (C alpha + beta), amino acid decarboxylase subgroup IV, alanine racemase, aminotransferase subgroup III and alliin lyase. Alanine racemase appears to be distantly related to decarboxylase subgroup IV. None of the PLP enzymes proved to be related with any other non-PLP protein in the database. The algorithm was also used to delineate the evolutionary pedigree within each family.

S09-21

CHANGES IN SUBSTRATE AND REACTION SPECIFICITY IN ACTIVE-SITE Arg→Lys ASPARTATE AMINOTRANSFERASE MUTANTS.

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The two active-site residues Arg386 and Arg292 of aspartate aminotransferase (AspAT) which bind the α and ω carboxylate group, respectively, of dicarboxylic substrates were replaced with lysine. The conservative Arg→Lys mutations markedly decreased the AspAT activity (k_{cat} = 0.5, 2.0 and 0.03 s^{-1}, respectively, for the R292K, R386K and R292K/R386K mutations; k_{cat} = 220 s^{-1} for the wild-type enzyme). The catalytic efficiency of all three mutant enzymes toward dicarboxylic substrates of any side-chain length was decreased. In contrast, the catalytic efficiency of AspAT R292K toward apolar substrates was not greatly affected. AspAT R292K decarboxylates L-aspartate to L-alanine 2000 times faster (k_{cat} = 0.27 s^{-1}) and catalyzes the racemization of L-aspartate, L-glutamate and L-alanine 3, 5 and 2 times faster, respectively, than the wild-type enzyme. The results show that the reaction specificity of a given B_6 enzyme is not only achieved by accelerating the specific reaction but also by preventing other potential side reactions.

S09-22

FUNCTIONAL DISPLAY OF *E. COLI* ASPARTATE AMINOTRANSFERASE (AspAT) ON FILAMENTOUS BACTERIOPHAGE M13 SURFACE

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As a first step towards directed evolution of B_6-dependent enzymes, we explored the feasibility of displaying *E. coli* AspAT on M13 surface. Direct insertion of the AspAT coding sequence (aspC) upstream to g8 or g3 (the genes encoding the major and a minor coat protein, respectively) was not successful. Fusion of Jun and Fos, each flanked by two cysteine residues (Crameri, R. and Suter, M. 1993) upstream to g8 or g3 and AspC, respectively, resulted in full-length fusion products. The Fos-AspC fusion protein translocated into the periplasm could be detected in cell lysate, but it was not efficiently incorporated into the phage particle as judged by phage-ELISA using polyclonal antiserum against AspAT. The incorporation was greatly enhanced, however, upon substitution of wild-type AspAT with its quintuple mutant in which all 5 cysteine residues had been mutated to alanine (Gloss, L.M., Planas, A., and Kirsch, J.F. 1992). The displayed mutant AspAT appeared to be properly folded, as AspAT activity could be measured in highly purified phage preparations. The strategy used for the successful display of *E. coli* AspAT may be applicable to other intracellular free sulfhydryl group containing proteins.

S09-23

PYRIDOXAL-5'-*P*-DEPENDENT CATALYTIC ANTIBODY

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Monoclonal antibodies were produced against the reduced Schiff base of pyridoxal-5'-phosphate (PLP) and L-lysine. The antibodies were screened with six different haptens, reduced Schiff bases of PLP and amino acids, for tight binding. Subsequently, they were screened for aldimine formation. Antibody 15A9 displayed several PLP-dependent catalytic activities such as α-proton exchange, β-elimination and transamination with different D-amino acid substrates. The antibody was capable of multiple turnovers. The results suggest routes toward generating cofactor-dependent catalytic antibodies.

S09-24

DIFFERENTIAL EFFECTS OF MOLECULAR CHAPERONES ON REFOLDING OF HOMOLOGOUS PROTEINS

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Three homologous aspartate aminotransferases (AspAT) with virtually identical spatial structures and pairwise amino acid sequence identities of > 40% differ markedly with respect to the yield of renaturation upon dilution from 6 M guanidine hydrochloride (mitochondrial << cytosolic < *E.coli*). The enzymes also respond differently to molecular chaperones. GroEL/GroES, the Hsp60 homolog of *E.coli*, increased considerably the yield of renaturation of mitochondrial AspAT and to a lesser extent that of its cytosolic counterpart, but not that of the *E.coli* enzyme. DnaK/DnaJ/GrpE, the Hsp70 system of *E.coli*, also increased the yield of renaturation of mitochondrial AspAT. Apparently, specific features in amino acid sequences and folding intermediates which are independent of the final secondary and tertiary structures determine the interactions of the proteins with the chaperone systems. The mode and rate of folding and the affinity for molecular chaperones might be features of the mature part of mitochondrial proteins determining their rate of importation into the organelles.

S09-25

Kinetics of peptide-binding and release in the DnaK/DnaJ/GrpE molecular chaperone system

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Molecular chaperones participate in the cellular protein folding pathway by preventing nonproductive folding. DnaK, the Hsp70 homolog of *E.coli*, acts in concert with the two co-chaperones DnaJ and GrpE which modulate its ATPase activity and thereby its peptide binding-release cycle. Using a short hydrophobic peptide, labeled with an environmentally sensitive fluorophore, and stopped-flow techniques, we obtained results that are interpreted with the following model of operation of the DnaK/DnaJ/GrpE chaperone system: (1) Binding of ATP rather than its hydrolysis induces a conformation of DnaK which binds the target peptide in a very rapid single-step process (k_{obs}= 6.8 s^{-1} at 50 nM substrate, 1 μM DnaK, 25°C) and with low affinity (K_d=5.1 μM). Equimolar concentrations of GrpE do not affect the binding. (2) Catalytic amounts of DnaJ convert DnaK·ATP to a second conformation which binds the target peptide in a slow two-step process with high affinity: k_{obs1}= 0.3 s^{-1}, k_{obs2}=0.02 s^{-1}, K_d=0.1 μM. This conformational change most likely results from the efficient conversion of DnaK·ATP to DnaK·ADP·P_i and prevents the release of the loosely bound target peptide from DnaK by reducing k_{off}.(3) ADP·P_i·DnaK·substrate forms a tight ternary complex with DnaJ (K_d=0.14 μM). (4) Binding of GrpE to the preformed ternary complex leads to very fast release of ADP·P_i and formation of a putative GrpE·DnaK·substrate·DnaJ intermediate (k_{obs}=17.4 s^{-1}) which after binding of ATP to DnaK dissociates and releases the substrate with k_{obs}=1.3 s^{-1}. DnaK·ATP is now available for a new cycle.

S09-26

Effect of potassium ions on the molecular chaperone activity of DnaK

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In DnaK, the Hsp70 homolog of *Escherichia coli*, we found K^+ to stabilize the chaperone·nucleotide complex, that forms on incubation of nucleotide-free DnaK with ADP or ATP. Na^+ was ineffective in complex stabilisation. The stabilizing effect of potassium ions is most likely due to their direct complexation, together with Mg^{2+}, to the bound nucleotide [Wilbanks, S.M. & McKay, D.B., 1995] The formation of this highly coordinated complex is required not only for the stimulation of ATPase activity but also for the peptide-induced increase in ATPase activity. The ATP-induced acceleration of binding of peptide ligands is about 20 times higher in the presence of K^+ than in the presence of Na^+. The ATP-induced release of peptide ligands is strictly dependent on the presence of K^+ and does not function at all when K^+ is replaced by Na^+. Thus, K^+ is an irreplacable metal cofactor of DnaK and very likely the other Hsp70s, being instrumental in signal transmission between the ATPase domain and the peptide-binding domain.

S09-27

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT SPINACH FERREDOXIN:THIOREDOXIN REDUCTASE

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Ferredoxin:thioredoxin reductase (FTR) is a 25.6 kDa Fe-S protein involved in the light-dependent enzyme regulation in oxygenic photosynthesis. It is composed of two dissimilar subunits, of about equal size. The catalytic subunit contains a redox-active disulfide bridge functional in the reduction of thioredoxins and a [4Fe-4S] cluster. The other subunit has no known catalytic function. *E.coli* cells have been transformed with a di-cistronic construction carrying, in phase and on the same DNA strand, the coding parts of the genes for both subunits. Fully functional FTR has been recovered from the bacterial cells. The recombinant protein has the same chromatographic and electrophoretic properties as native FTR and shows the typical absorption spectrum of FTR. These results indicate that the [4Fe-4S] cluster has been inserted into the catalytic subunit and that the two dissimilar subunits are correctly assembled.
(SNF 31-37725.93)

S09-28

ROLE OF CYS73 OF SPINACH THIOREDOXIN *f* IN DIMER FORMATION

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Spinach thioredoxin *f* is a monomeric globular chloroplast protein of 12.5 kD. It participates in the light regulation of photosynthetic CO_2 assimilation by activating key chloroplast enzymes like fructose 1,6-bisphosphatase (FBPase). Besides the two Cys residues constituting the redox active disulfide bridge, present as active site in all thioredoxins, thioredoxin *f* has an accessible, third Cys at position 73, close to the active site. Using site directed mutagenesis we could show, that Cys73 is *in vitro* responsible for a homodimer formation observed under oxidative conditions. Under the same conditions also a heterodimer between thioredoxin *f* and its target enzyme FBPase can be obtained. It remains to be shown whether these observations have a physiological significance under conditions of oxidative stress. (SNF 31-37725.93)

S09-29

TOPOLOGY OF THE MOUSE SECRETORY COMPONENT UPON ASSOCIATION WITH DIMERIC IgA

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It is not clear what features secretory component (SC) contributes to secretory IgA, the main effector of mucosal immunity. SC is composed of five immunoglobulin (Ig)-like domains, domain I being crucial for non-covalent binding, while domain V is involved in partial disulfide bridging to IgA. A 3-D model of domains II and III of mSC predicts beta-sheets connected by loops, based on a sequence alignment with members of the Ig superfamily of established structure. To address structure-function aspects of SC, mouse (m) SC constructs expressed in mammalian cell culture can be associated *in vitro* with purified dimeric IgA obtained from a hybridoma (ZAC3, anti-*Vibrio cholerae* LPS). ELISA and co-immunoprecipitation assays allow to assess the surface availability of the loops in free *versus* IgA-bound mSC. The topology of IgA-reassociated mSC was tested using monospecific Abs raised against selected loops of mSC. Conversely, these same loops in recombinant mSC were individually replaced with a FLAG epitope, which is tractable using a commercial MAb. Loops whose replacement is compatible with high affinity binding to IgA are chosen to introduce protective linear epitopes from the pathogen for a combined passive/active immunization strategy using redesigned sIgA.
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S09-30

Fructose-bisphosphate aldolases: demonstration of homology and evolutionary relationships

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Fructose - bisphosphate aldolase is a key glycolytic enzyme catalysing a variety of closely related aldol cleavage and condensation reactions. Two forms of this widely distributed enzyme are found in nature designated as class I and class II aldolases. Class II enzymes utilize a divalent metal ion which acts as an"electron sink" in the catalytic mechanism. Class I aldolases or Schiff's base forming aldolases are found in vertebrates in a number of isoforms. A total of 27 amino acid sequences of the key glycolytic enzyme class I fructose-bisphosphate aldolase have been examined for evolutionary relationships and amino acid homology. All aldolase sequences were aligned by using standard methods for sequence comparison based on hydropathy patterns and regions of secondary structure. Phylogenetic analyses were made from the aligned amino acid sequences together with one- and three- dimensional profile analyses to establish evolutionary and structural relations. The results reveal evolutionary relationships differing in part from previously reported views in that several of the known isoforms of aldolase seem to be present in most species examined. The type B isoform showed itself to be the first aldolase to have emerged in the evolution of the vertebrates. Phylogenetic tree calculation and multialignment results will be presented proposing a revised view of class I fructose-bisphosphate aldolase evolution and its relationship to class II aldolases.