G1–Molecular Chaperones

G1-001
Stress and misfolded proteins: modulators of neurodegenerative diseases and longevity

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Misfolded proteins, aggregates, and inclusion bodies are hallmarks of the cytopathology of neurodegenerative disorders including Huntington's disease, Amyotropic lateral sclerosis, Parkinson's disease, Prion diseases, and Alzheimer's disease. The appearance of proteins with altered folded states is regulated by the protein folding quality control machinery and age-dependent. We have identified an unexpected molecular link between metabolic state, accumulation of damaged proteins, the heat-shock response and chaperones, and longevity. Mutations (age-1, daf-2) in the insulin-like signaling (ILS) pathway in *C. elegans* leading to longevity results in the suppression of polyglutamine toxicity and aggregate formation. Because overexpression of HSF-1, a known regulator of chaperone networks and quality control, was also shown to suppress polyglutamine aggregation, we examined whether HSF-1-regulated lifespan. Downregulation of hsf-1 by RNAi in neurons and muscle cells suppressed longevity, which reveals a new molecular link between longevity and stress resistance. To identify other modifiers of protein quality control, we screened transgenic polyglutamine-expressing strains using genome-wide RNAi to identify genes that regulate polyglutamine aggregation. Nearly 200 genes were identified defining a ''protein quality control proteome'' corresponding to five principal classes of polyglutamine regulators: genes involved in RNA metabolism, protein synthesis, protein folding, protein degradation, and those involved in protein trafficking. We propose that each of these classes represents a molecular machine that collectively comprises the protein homeostatic buffer that responds to the expression of damaged proteins to prevent their misfolding and aggregation.

G1-002
Chaperone machines of the cytosol

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The ensemble of molecular chaperones constitutes the cellular system that assists folding and assembly of newly synthesized proteins, translocation of unfolded proteins across membranes, as well as refolding and degradation of misfolded and aggregated proteins. In the *Escherichia coli* cytosol, the ribosome-associated trigger factor assists the first steps in the co-translational folding of nascent polypeptide chains. The major Hsp70 chaperone, DnaK, uses the energy of ATP and the assistance by the DnaJ and GrpE co-chaperones, to prevent aggregation and support refolding of damaged proteins. DnaK furthermore cooperates with the AAA + ATPase, ClpB, to solubilize and refold aggregated proteins. The remarkable remodeling activity of ClpB is essential for cell survival under severe heat stress. This seminar will describe our current knowledge of the working mechanism of these chaperone machines.

G1-003
The substrate spectrum of the eucaryotic chaperonin TRIC/CCT revealed by genomic approaches

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The cytosolic eukaryotic chaperonin TRiC/CCT (for TCP1 ring complex or chaperonin-containing TCP1) has an important role for folding in the cell, as evidenced by its essentiality for life. Initially, this requirement was thought to be a consequence of an exclusive role in folding the cytoskeletal proteins actin and tubulin. However, an increasing number of reports indicate that TRiC interacts with a broader spectrum of proteins than first proposed. To systematically determine the contribution of TRiC to *de novo* folding, we have undertaken both proteomic and genomic approaches to isolate a comprehensive set of TRiC-interacting proteins. TRiC associated with approximately 5–8% of newly synthesized proteins that were surveyed, encompassing a wide spectrum of proteins involved in many cellular processes. A common feature of the TRiC-interacting set is an enrichment of large multidomain proteins. Further, these proteins generally contained larger domains. Analysis of various chemical and structural properties common to the TRiC-interacting polypeptides revealed several interesting trends that will be discussed. It is tempting to suggest that the features common to this protein class may contribute to their association with TRiC.

G1-004
Disulfide bond formation during protein folding in the endoplasmic reticulum

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Protein folding in the endoplasmic reticulum (ER) is assisted by a large number of chaperones and folding enzymes, some of them members of conserved chaperone families, others committed to specialized reactions in the ER. Up to now, the number of mammalian ER proteins suspected to catalyze disulfide bond formation or isomerization alone already is 22, with more to be identified. Our research focuses on disulfide bond formation and the oxidoreductases that assist the process during folding of our model proteins Influenza virus hemagglutinin, HIV-1 Envelope glycoprotein, and the low-density lipoprotein receptor. Oxidoreductases may affect both efficiency and rate of folding and disulfide bond formation, depending on cellular redox conditions, and their relative expression ratios.

G1-005
Navigating the chaperone network: an integrative map of physical, genetic, and chemical–genetic interactions mediated by the yeast Hsp90 chaperone system

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Physical, genetic, and chemical–genetic interactions centered on the conserved chaperone Hsp90 were mapped at high resolution in yeast using systematic proteomic and genomic methods. Physical interactions were identified using genome-wide two-hybrid screens combined with large-scale affinity purification of Hsp90-containing protein complexes. Genetic interactions were uncovered using synthetic genetic array technology and by a microarray-based chemical–genetic screen of a set of approximately 4700 viable yeast gene deletion mutants for hypersensitivity to the Hsp90-inhibitor geldanamycin. An extended network, consisting of 198 putative physical interactions and 451 putative genetic and chemical–genetic interactions, was found to connect Hsp90 to cofactors and substrates involved in a wide range of cellular functions. Two novel Hsp90 cofactors, Tah1 (YCR060W) and Pih1 (YHR034C), were also identified. These cofactors interact physically and functionally with the conserved AAA+-type DNA helicases Rvb1/Rvb2, which are key components of several chromatin-remodeling factors, thereby linking Hsp90 to epigenetic gene regulation.

G1-006
Ribonucleic acid chaperones and their significance during ribosome biogenesis

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Chaperones are generally viewed as proteins that facilitate proper folding of other proteins often by preventing aggregation of folding intermediates. Another important class of chaperones is ribonucleic acid (RNA) chaperones. RNA chaperones are proteins that facilitate conformational changes of RNA molecules to assist the assembly and disassembly of RNA–RNA and RNA–protein interactions during dynamic processes such as viral replication, pre-mRNA splicing or ribosome biogenesis. During ribosome biogenesis ribosomal RNAs (rRNAs) are transcribed as a large precursor-rRNA (pre-rRNA) and accurately processed by an efficient mechanism, ultimately producing mature ribosomal subunits. This mechanism in eukaryotes requires the formation of base pair interactions between the pre-rRNA and processing small nucleolar RNAs (snoRNAs) presumably to help the proper folding of the rRNA and to guide target site selection of the processing endonucleases. Even though RNA duplexes can form spontaneously, cells often use proteins to stimulate hybridization for various reasons: the site of hybridization is buried, the hybridization is too slow or the duplex is unstable. In this study, we focus on the formation of two short duplexes between the U3 snoRNA and complementary sites of the pre-rRNA. The U3 snoRNA and its associated proteins, designated the small subunit processome (SSUP) [1], assists the first essential processing events during ribosome biogenesis, the release of the small subunit rRNA precursor from the pre-rRNA. The expected role of the pre-rRNA-U3 snoRNA hybrids is to guide the SSUP to the target cleavage sites on the pre-rRNA. We demonstrated that an essential protein of the SSUP, Imp4p from *Saccharomyces cerevisiae*, is able to mediate duplex formation *in vitro* at both essential U3 snoRNA-pre-rRNA base pairing sites presumably to help recruit the SSUP to its target, the pre-rRNA [2]. At one site Imp4p stabilizes an otherwise unstable hybrid, whereas at the other site Imp4p acts as a chaperone to unmask the relevant nucleotides prior to hybridization. To provide evidence that the annealing activities of Imp4p observed *in vitro* is needed for the proper docking of the SSUP onto the pre-rRNA we are pursuing *in vivo* studies that will exploit an Imp4p mutant. This mutant retains binding to the U3 snoRNA *in vitro* and is thus expected to assemble into the SSUP. Importantly, the mutant fails to mediate formation of U3 snoRNA-pre-rRNA duplexes *in vitro*

and thus is expected to be defective in rRNA processing and perhaps cell growth. As a first step toward probing the molecular mechanism of the annealing activity we will determine the kinetic rate constants and rate order of these annealing activities. We will correlate *in vitro* catalytic efficiency to that expected *in vivo*.

References

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G1-007P
Modulation of tropoelastin co-acervation by FKBP65

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FKBP-65 belongs to the immunophilin family of proteins, which bind the immunosuppressive drugs, FK506 and rapamycin and exhibit peptidyl prolyl isomerase (PPIase) activity. Based on cell biological data on the co-localization of FKBP65 with tropoelastin (TE) in the ER and in the early secretory compartments, Davis et al. (*J Cell Biol* 1998; **140:** 295) proposed that FKBP65 may act as a molecular chaperone for TE. Using recombinant mouse FKBP65, we show here that, under physiological pH, rFKBP65 significantly enhances the rate and extent of *in vitro* co-acervation of TE, as monitored by turbidity increase at 300 nm. This effect is also seen in the case of TE model polypeptides, which have sequences corresponding to alternating hydrophobic and cross-linking domains of TE. These results suggest that FKBP-65 may act as a TE-specific molecular chaperone *in vivo* by modulating the co-acervation of TE which is crucial for elastin fibre formation.

G1-008P
Effect of Hsp90 inhibitors, geldanamycin, 17-allylamino-17-demethoxygeldanamycin and curcumin on human neuroblastoma cells, IMR-32

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Hsp90, the 90 kDa heat-shock protein is an abundant molecular chaperone involved in various cellular processes such as cell proliferation, differentiation and apoptosis. Disruption of Hsp90 chaperone function with geldanamycin (GA) and its analogue 17-allylamino-17-demethoxygeldanamycin (17AAG) has evolved as a recent antitumour therapy. Curcumin (diferuloylmethane), a yellow pigment isolated from *Curcuma longa* L. is a known antioxidant, anti-inflammatory and anticarcinogenic agent. We have studied the sensitivity of these antitumour agents on human neuroblastoma cells, IMR-32. Neuroblastoma cells, when treated with 15 μM curcumin, exhibit a differentiation like morphology. Hsp90 inhibitors at a concentration of 2 μM show similar morphology with extensive neurite out growth. Florescence-activated cell sorting (FACS) analysis of drug-treated cells shows that curcumin treatment leads the cells to S phase whereas GA/17AAG treatment arrests them at G1 phase. GA or 17AAG supercede the curcumin effect and arrest cells in G1 phase. This G1 arrest is associated with the activation of extracellular-regulated kinases 1 and 2 (ERK1/2) and deactivation of MEK1/2. Treatment of IMR-32 cells with curcumin alone have no effect on cyclin D1, the G1 cyclin, but inhibitors or inhibitors in combination with curcumin results in the degradation of cyclin D1 and increase in p21Waf levels. Hsp90 inhibition with GA/17AAG treatment leads to upregulation of Hsp70 and Hsp27, and tumour suppressor protein, p53. However, curcumin either by itself or in combination with GA/17AAG inhibits the upregulation of Hsps and p53. Cells treated with curcumin and 17AAG on supplementing with 50 ng/ml of epidermal growth factor (EGF) recovered and entered cell cycle whereas GA-treated cells eventually undergo apoptosis. Our results suggest that human neuroblastoma, IMR-32 cells respond differently to curcumin, 17AAG and GA.

G1-009P
Functional characterization of heat-shock protein 70 homologues from *Agrobacterium tumefaciens*

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Molecular chaperones of the heat-shock protein 70 family (Hsp70; called DnaK in prokaryotes) play an important role in the folding and functioning of proteins in the cell. Hsp40 (DnaJ in prokaryotes) is an Hsp70 co-chaperone that stimulates the ATPase activity of Hsp70. Two *dnaK* genes from the Gram-negative plant pathogen *Agrobacterium tumefaciens* were amplified and the DnaK proteins (*Agt* DnaK1 and 2) were over-produced as His-tagged proteins in *Escherichia coli*. Complementation assays were used to determine if *Agt* DnaK1 and 2 can functionally replace *E. coli* DnaK *in vivo* using an *E. coli* mutant strain an *E. coli* deletion strain (characterized by very low DnaJ levels). The results have shown that *Agt* DnaK1 was able to functionally replace *E. coli* DnaK in the *E. coli* mutant strain and partially complement for the lack of *E. coli* DnaK in the deletion strain, which could be attributed to the low levels of DnaJ in the deletion strain. However *Agt* DnaK2 was unable to complement in either the *E. coli* mutant strain or deletion strain. *Agt* DnaK1 and 2 will be further characterized using an *E. coli* DnaK deletion strain which has normal DnaJ levels. Based on these results it would appear that *Agt* DnaK1 is a homologue of *E. coli* DnaK, while the function of *Agt* DnaK2 is unknown. Further *in vitro* characterization of these proteins will be done using refolding assays and ATPase assays. These molecular chaperones will ultimately be used in protein biotechnology to refold aggregation-prone enzymes from *A. tumefaciens*.

G1-010P
Importance of the quaternary structure in the chaperon function of small heat-shock proteins

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Small heat-shock proteins are low-molecular mass proteins, abundant in all kingdoms of life. Their role is to maintain substrate proteins in folding-competent state. These small heat-shock proteins generally form large multimeric complexes; the oligomers usually have a dynamic quaternary structure. The oligomerization is a prerequisite for the chaperone function, but the

relationship between the oligomer size and the chaperone activity is not well understood. In this work, we examined two members of this family: bovine alpha-crystallin, and a prokaryotic protein, HSP 16.5 from *Methanococcus jannaschii* (MjHSP 16.5). Pressure is an adequate tool for studying this question, because moderate pressure generate elastic changes in the protein, and affects mostly the quaternary structure. Both cases we observed that the chaperone activity of these proteins could be enhanced by short pressure treatment at 100–400 MPa. We studied by various spectroscopic methods the underlying structural changes. The results showed that mostly the quaternary structure altered during this treatment, and it should be the key factor in the chaperone activity. Our results showed that the enhancement of the chaperone activity does not require the increase of the oligomer size as was assumed earlier, and that the pressure is a very powerful tool studying the function of oligomeric proteins.

G1-011P
HspB8 overexpression inhibits mutated huntingtin aggregation

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In mammals small heat-shock proteins (sHsp) comprises 10 members, many of which are considered as molecular chaperones based on demonstrated *in vitro* activity. Whether these proteins behave as chaperones *in vivo* remains unclear. Here, we investigated the effect of HspB8 on the expression of Htt43Q, a fragment of the huntingtin protein containing a 43 glutamine repeat. Upon transient transfection, the polyglutamine protein accumulated in cells as perinuclear inclusions. Co-transfected with Htt43Q, HspB8 was in many cases localized within the Htt43Q inclusions but in most cells efficiently blocked their formation. Biochemical analyses indicated that only HspB8, but not other sHsps (e.g. Hsp27), severely inhibited the accumulation of SDS-insoluble Htt43Q. The HspB8 activity was identical to that obtained by overexpressing Hsp40. Moreover, treatment with the proteasomal inhibitor MG132 and the autophagic inhibitor 3-methyladenine induced the accumulation of Htt43Q only in the SDS-soluble fraction. These results suggest that HspB8 acts as a molecular chaperone maintaining Htt43Q in a soluble state competent for rapid degradation in the cells. HspB8 also prevented the insolubilization of AR65Q, androgen receptor with a 65 glutamine repeat, indicating a general chaperone activity of HspB8 against destabilized polyglutamine proteins. Analyses of Hsp27–HspB8 chimeric proteins suggested that the C-terminal domain of HspB8 contains the sequence necessary for the chaperone activity. Recently, two missense mutations at lysine 141 of HspB8 have been associated to hereditary motor neuropathies. Both mutations slightly reduced the chaperone activity.

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G1-012P
Antibodies reactive to heat-shock protein 90 induce oligodendrocyte precursor cell death

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The lack of remyelination is one of the causes of progressive loss of neurologic function in demyelinating diseases. There are not effective ways to promote remyelination in the central nervous system (CNS) when it has been affected. It has been observed that surviving oligodendrocytes in an area of demyelination do not contribute to remyelination, which is only developed by oligodendrocyte precursor cells (OPCs). Recent evidence suggests that these cells are extremely efficient at myelin repair, either spontaneously or after transplantation into the CNS. The results obtained in our laboratory, using OPC cultures, demonstrate that heat-shock protein 90 (Hsp90) is expressed on the surface of these cells. Cell cultures treated with anti-Hsp90 antibody present a selective death of OPC. This cell death depends on complement activation, with a significant reduction of OPC population. As a consequence of OPC death, it was not observed the existence of oligodendrocytes in cultures treated with anti-Hsp90 antibody. Further, complement inhibition by either heating or addition of specific inhibitors of complement system, allowed OPC survival in the presence of anti-Hsp90 antibodies and their maturation to oligodendrocytes. Our findings support the idea that long-term exposure to anti-Hsp90 antibodies and complement activation could contribute to a reduction in the OPC population and limit remyelination.

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G1-013P
Mapping of functional residues in the Hsp70/Hsp90 organizing protein, mSTI1

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The murine stress-inducible protein 1 (mSTI1) is a homologue of the human Hsp70/Hsp90 organizing protein (Hop), which mediates the formation of the Hsp70/STI1/Hsp90 chaperone heterocomplex. mSTI1 interacts with Hsp70 and Hsp90 through its tetratricopeptide repeat motifs (TPR) namely, TPR1 and TPR2A domains, respectively. The role of a third TPR domain, TPR2B, has not yet been elucidated. mSTI1 is an *in vitro* substrate of cell cycle kinases, casein kinase II (CKII) and cdc2 kinase, which may be involved in subcellular localization of this protein. The phosphorylation sites for CKII and cdc2 kinase are found upstream of the TPR2A domain. Moreover, a putative nuclear localization signal (NLS) has been identified which overlaps with TPR2A domain. Site-directed mutagenesis and surface plasmon resonance (SPR) spectroscopy were used to map residues in mSTI1, which are critical for its functionality as a Hsp70/Hsp90 co-chaperone. A construct of TPR2B and the C-terminal end of mSTI1 was shown to interact with Hsp70 and Hsp90 thereby implicating TPR2B and/or the C-terminal domain in the formation of the Hsp70/STI1/Hsp90 heterocomplex. Cdc2 and CKII – phosphorylation mimics of mSTI1 gave dissociation constants within the same order of magnitude as the unmodified protein. However, the cdc2-phosphorylation mimic (mSTI1-T198E) showed a partial decrease in interaction with Hsp90, compared with the unmodified protein. Mutations within the proposed NLS abrogated mSTI1–Hsp90 interactions, leading to the conclusion that K237, K238 and K239, which are predicted to be important for functioning of the NLS, are also involved in mSTI1–Hsp90 interactions. These studies implicate a possible link between the mechanism of mSTI1–Hsp90 interactions and nuclear localization of mSTI1. In addition, residues involved in the mSTI1/Hsp70/Hsp90 interactions are not restricted to TPR1 and TPR2A domains but may include TPR2B domain.

G1-014P
Protein interactions and fluctuations in a proteomic network using an elastic network model

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A set of protein conformations are analyzed by normal mode analysis. An elastic network model is used to obtain fluctuation and cooperativity of residues with low amplitude fluctuations across different species. Slow modes that are associated with the function of proteins have common features among different protein structures. We show that the degree of flexibility of the protein is important for proteins to interact with other proteins and as the species gets more complex its proteins become more flexible. In the complex organism, higher cooperativity arises due to protein structure and connectivity.

G1-015P
Expression and purification of ST14, a tumor metastasis-related protein, and its activity assay

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ST14 (suppression of tumorigenicity 14) is one of the type II transmembrane serine proteases that association with the process of tumor metastasis. The C-terminal catalytic region (900 bp) of ST14 gene was cloned into the expression vector pGEX-4T-2 and the positive plasmid pGEX-4T-2-ST14 was transformed into *Escherichia coli* BL21, then cultured and induced with isopropyl-beta-D-thiogalactopyranoside (IPTG).The chaperonin GroEL was found to be tightly associated with the fusion protein and co-purified with it by regular glutathione-S-transferase (GST) affinity chromatography. A method for the removal of contaminating GroEL from GST–ST14 fusion protein was described, the purity of product was 96.2%. Enzyme activity assay indicated that the purified fusion protein had higher serine protease activity.

G1-016P
Protein stabilization by osmolytes from hyperthermophiles: effect of mannosylglycerate on the thermal unfolding of sthaplylicoccal nuclease studied by picosecond time-resolved fluorescence and calorimetry

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Compatible solutes are low-molecular weight molecules accumulated by microorganisms upon growth under supraoptimal temperature or salinity that protect cellular components against stressful environmental conditions. Hyperthermophiles accumulate mainly negatively charged solutes, like mannosylglycerate (MG), in opposition to mesophiles that accumulate non-charged or zwitterionic solutes. *In vitro*, MG exerts a strong protective effect on enzymes during freeze-drying or heating. It also stabilizes the protein structure at high temperature or high guanidinium chloride concentration. In the present study, we studied the effect of MG on nuclease A from *Staphylococcus aureus* (SNase) by Differential Scanning Calorimetry and by Time-resolved Fluorescence Spectroscopy using the single tryptophan fluorescence as an intrinsic probe for the protein structure. Quantum yield, maximum emission wavelength and fluorescence excited state lifetime of tryptophan depend on the environment polarity and temperature. The study of these parameters can provide insight into the protein-unfolding pathway. The fluorescence intensity decays of SNase are weighted sums of the decays of the tryptophan residue in the folded and unfolded forms of the protein. The pre-exponential coefficients can be used to evaluate the molar fractions of the protein forms. Hence, direct determination of equilibrium constants of unfolding was carried out and a thermodynamic analysis was performed. The presence of 0.5 M MG caused an increase of 7 °C in the SNase melting temperature and a twofold increase in the unfolding heat capacity. However, the nature of protein states along unfolding was not altered in the presence of MG, denoting that the unfolding pathway was unaffected. In molecular terms, stabilization is interpreted as resulting from destabilization of the denatured state caused by preferential exclusion of the solute from the protein hydration shell upon unfolding, and stabilization of the native state by specific interactions.

G1-017P
Regulation of the accessibility and stability of substrate–complexes by ionic interactions in DnaK

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Hsp70 molecular chaperones are an ubiquitous and conserved family of proteins that participate in multiple cellular processes including protein folding/unfolding, protein translocation across membranes, assembly/disassembly of protein complexes and refolding of protein aggregates. All these Hsp70 functions rely on their capacity to bind short hydrophobic segments of proteins in a nucleotide-regulated manner. Depending on the ADP- or ATP-bound nucleotide, the C-terminal α-helical "lid" subdomain of Hsp70 proteins adopts different conformations that modulate both accessibility and stability of Hsp70 substrate–complexes. In order to further characterize the conformations that the α-helical subdomain can sample, we have constructed mutants of the bacterial homologue *Escherichia coli* DnaK. Our results help to understand the structural requirements that module the α-helical subdomain movements and how they regulate, at least in part, substrate binding cycles in Hsp70 proteins.

G1-018P
Induction of heat-shock proteins by heavy metal exposure and temperature stress in fish heart

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Heat-shock proteins (HSPs) are essential for cells under physiological conditions. They are needed even more after exposure to a

wide variety of stressors, such as heat shock, heavy metals, or almost any sudden change in the cellular environment, which induces protein damage. Our studies focused on *hsp70, hsc70-1* and *hsp90α*. Their expression was followed after hypo- and hyper-thermia, and Cd challenge, in the cardiac cells of common carp (*Cyprinus carpio*), in whole animal experiments. High level of *hsc70-1* transcript was detected in unstressed cells, while the concentration of *hsp70* and *hsp90α* mRNAs was around the limit of detection. The inducibility of these selected stress genes was gene- and stress-specific: A 14 °C increase of temperature for 0.5 h resulted in a 10- to 12-fold enhanced expression of both *hsp70* and *hsp90α*. The elevated *hsp70* level was not significantly increased further during 3 h of hyperthermia and it also persisted during a 1 h recovery period. However, the high *hsp90α* mRNA level was markedly decreased during the recovery. *Hsp70* and *hsp90α* mRNA levels were characterized by a 10- to 12-fold and a 20-fold increase, respectively, after 1 h of hypothermia (Δ7 °C) followed by 1 h of recovery. Neither hypo- nor hyper-thermia had a marked effect on *hsc70-1* expression, only an 1.5- to two-fold induction was detected after both treatment. Cd treatment resulted in a 20- to 25-fold induction of *hsp70*, while it had no detectable effect on the expression of *hsc70-1* and *hsp90α*.

G1-019P
Cytoprotective role of chaperones DnaK and GroEL during inclusion body formation

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In bacteria, protein aggregation as inclusion bodies is a common and relevant phenomenon during recombinant protein production. In eukaryotic cells, the formation of inclusion bodies or amyloid protein aggregates is toxic for the cells, and it causes many neurodegenerative diseases such as Alzheimer and Huntington's disease. Nevertheless, bacterial inclusion bodies had not been studied in the precise cytotoxicity context. In the present study, we have studied cell viability and culture growth of wild-type strains the MC1061 and the MC4100 and the MC4100 derivatives GroEL$^-$, DnaK$^-$, Lon$^-$, and ClpA$^-$, expressing the inclusion body-forming VP1LAC protein. VP1LAC is a beta-galactosidase fused with the foot-and-mouth disease VP1 capsid protein. As a control, the parental beta-galactosidase has been used, since this protein is completely soluble and does not aggregate under physiologic conditions. The production of VP1LAC, although not of the control beta-galactosidase, is toxic in DnaK- and GroEL-deficient strains, but not in wild-type MC1061 and MC4100 and its derivatives Lon$^-$ and ClpA$^-$. The presented data can be understood in the context of the quality control system, where the chaperone DnaK and the chaperonine GroEL could act as cytoprotective elements.

G1-020P
Biological and conformational state of soluble recombinant proteins

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In *Escherichia coli*, the chaperone DnaK is an important folding modulator and, in its absence, the bacterial production of foreign recombinant proteins usually results in the formation of large inclusion bodies. But, even under these conditions, an important part of these polypeptides still remains in the soluble fraction. In this study, we have explored the status of recombinant proteins in the soluble fraction produced in absence and presence of the chaperone DnaK. The model recombinant protein has been VP1LAC, an amino-terminal beta-galactosidase fusion protein, carrying the VP1 capsid protein of food-and-mouth disease virus that dramatically reduces the solubility of the whole fusion. We have observed that VP1LAC-specific activity in DnaK-mutants is lower than when produced in wild type cells. However, in both strains, the specific activity in soluble and insoluble fraction is quite similar. Therefore, we decided to further explore the status of the soluble fraction by analysing the denaturation kinetics of this model protein. Our results reveal that, at the early exponential phase of the culture, thermal stability of VP1LAC is about twofold lower than when produced in wild type cells. However, when the production of VP1LAC is triggered at the beginning of the stationary phase, its stability is enhanced about two times irrespectively of DnaK. This stabilization results in an increase in the specific activity of soluble VP1LAC. These results suggest the possible existence of misfolded proteins forming soluble aggregates. We are probing the biological-conformational state of the soluble fraction of misfolding-prone recombinant proteins such us VP1LAC and a new GFP-derived construction, VP1GFP.

G1-021P
The macrophage migration inhibitory factor exhibits chaperone and anti-chaperone activities

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Macrophage migration inhibitory factor (MIF) is a ubiquitous regulatory cytokine of 115 amino acids having diverse immunological and neuroendocrine functions. Although MIF is known to be released into the circulation from secretory granules of anterior pituitary or directly from immune cells as a consequence of stress, the participation of MIF in heat stress-induced aggregation of proteins has not yet been reported. We provide here the first evidence that MIF possesses chaperone-like properties. At substoichiometric concentrations, MIF stabilizes thermally denatured malate dehydrogenase and glycogen phosphorylase b, prevents aggregation of these model proteins under heat stress and facilitate the recovery of their activity *in vitro*. Protein aggregates can be easily resolubilized. MIF seems to have some of the essential features of a chaperone such as surface hydrophobicity and is shown to exist in the form of large oligomers. Similar chaperone-like effects were also observed in the presence of partially purified brain extract containing, besides MIF, a number of ubiquitous hydrophobic low-molecular weight proteins identified by N-terminal microsequence analysis. Being highly stable and hydrophobic, MIF in combination with other proteins of similar properties may comprise a family of constitutively expressed "small chaperones" that counteract the early onset of stress, around physiological conditions (at 40–42 °C), when heat-shock proteins are not abundant. However, at higher temperatures (43–48 °C) MIF displays the ability to facilitate aggregation, termed anti-chaperone activity. This behaviour is associated with the appearance of hetero-aggregates that contain both MIF and substrate proteins.

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G1-022P
Some molecular characteristics of RNA-binding by Hsp70 and its homologues: is there a physiological relevance?

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Intriguing new functions of the ubiquitous 70-kDa molecular chaperone family members have been revealed in the past years. Among these, specific RNA recognition and binding implicated Hsc70/Hsp70 as RNA chaperones within multiple processes of eukaryotic RNA metabolism. We have earlier identified the molecular domains involved in RNA-binding and, recently, characterized in details the affinity and specificity of this molecular interaction using various deletion mutants of Hsp70. We have provided evidence that the N- and C-terminal domains of the molecule cooperate in determining RNA-recognition commitment and binding fidelity, and that conformationalrestrictions of the chaperone lead to loss of RNA-binding specificity. Furthermore, we have demonstrated distinct RNA-binding properties for various homologues of Hsp70 via sequence specificity, ribopolymer sensitivity as well as north-western analysis. Investigating DnaK the *Escherichia coli* homologue in RNA-binding analyses identified a regulatory role for its co-chaperones DnaJ and GrpE, a process with resemblance to the classical protein-binding cycle of this class of chaperones. Using P388 mouse macrophage cell line, here we show that wild type but not any of the truncated mutants of Hsp70 is effectively taken up by these antigen-presenting cells (APCs). Moreover, when co-administered with Cy5-labelled RNA, Hsp70 is capable of facilitating cytoplasmic delivery of RNA into APCs as revealed by fluorescent and confocal microscopy. Potential regulatory involvement of this RNA-binding capability by members of the 70-kDa family of molecular chaperones is discussed in various aspects of RNA metabolism with special emphasis on mRNA stability, localization and degradation.

G1-023P
Accumulation of heat-shock proteins 70 in seven cultivars of alfalfa after heat-shock treatment

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Heat-shock proteins (HSP) synthesis is known to be activated after heat stimuli in several organisms including plants. Their role *in vivo* is mainly directed both in keeping cellular homeostasis and in helping protein folding and refolding. Recently mammalian HSPs have been shown to have strong potential in improving immune response to pathogen attack and against tumours. Besides they have strong adjuvant property. Human and plant HSPs share high sequence similarity and they show the conservation of some important biological function *in vitro*. On this base plant HSPs could be exploited as an alternative source of natural adjuvants. In plants HSPs are activated transiently after heat shock and their expression level remains high up to several minutes after induction. The aim of our work was to follow the increasing of HSP70 in alfalfa (*Medicago sativa* L.) leaves following heat-shock treatment and its dependence from the genetic background. This study has two main goals: (i) to correlate the level of protein with heat tolerance, (ii) to find the optimal conditions in terms of time and temperature to increase HSP70 levels and subsequently purify this protein from the green mass. Experiments were carried out on seven different alfalfa cultivars using five different temperatures of heat-shock treatment. Heat shocked leaves were harvested and frozen. Cytosolic proteins were extracted and analysed through SDS-PAGE and Western blot. The response of different varieties to heat-shock treatment will be discussed.

G1-024P
Molecular chaperones alleviate misfolding of cystathionine beta-synthase mutants

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Conformational diseases result from misfolding and aggregation of mutant proteins. This group of diseases may include homocystinuria due to cystathionine beta-synthase (CBS) deficiency. Interestingly, an incorrect folding of newly synthesized polypeptides may be prevented by various chaperones. In this study, we provide more evidence on conformational changes in a larger panel of 25 mutants, which are localized in different domains of the CBS enzyme. The mutants were expressed in *Escherichia coli* in the presence and absence of different types of chaperones. Conformation of mutants was monitored by non-denaturing electrophoresis followed by Western blotting, by staining for heme and also indirectly by assessing their catalytic activity. So far, five model mutants were co-expressed with panel of bacterial chaperones (dnaK, dnaJ, grpE, groES and groEL) to test whether preventing misfolding may reverse their aggregation. Co-expression of mutants A114V, E176K and del exon 12 with groES-groEL indeed resulted in a substantial increase of catalytic activity to 90, 40 and 10% of wild type control, and to formation of some tetramer, while there was only small effect on the three remaining mutants. Our ongoing study will determine the effect of molecular chaperones on the rest of CBS mutants in different protein domains. In conclusion, our results suggest that some CBS mutants expressed in *E. coli* may be partially stabilized in active conformational state by molecular chaperones and that homocystinuria is another example of conformational disorders.

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G1-025P
Chaperone levels and function in the ageing immune system: the effect of zinc

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Throughout life, adaptive responses of the organisms drastically decrease, resulting in the accumulation of molecular damage, leading to ageing and finally to death. The protein homeostasis hypothesis of ageing focuses on the disturbances of protein metabolism as a possible cause and consequence of ageing. The major protective mechanism, at the level of the protein kingdom, is the cellular stress response. In course of this, protein denaturation evokes the induction of molecular chaperones, which, in turn, prevent further denaturation, aggregation and govern protein renaturation or direct them to disposal. Moreover, the major chaperones fulfil a vital housekeeping role by ensuring the proper folding of otherwise unstable signalling client molecules. Zinc is a potent inducer of the stress response.

Since immune function is a key determinant of longevity, and is greatly affected by optimal zinc level, we set out to investigate chaperone function in immune cells of young, aged and successfully aged (centenarian) people with different zinc status. Two important parameters affecting chaperone function are their steady-state level and general chaperone activity, therefore methods are being developed to determine heat-shock protein 70 (Hsp70) and 90 (Hsp90) mRNA and protein levels as well as *in vivo* refolding of a model substrate. Moreover, specific client chaperone interaction is being tested with a limited subset of possible clients related to ageing and zinc metabolism, in which cases the chaperone connection was not shown, yet. Current status of our project will be shown.

G1-026P
Heat-shock response is associated with decreased production of interleukin-6 in murine aortic vascular smooth muscle cells

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Heat shock has been known to change cellular responses to noxious stimuli by inducing heat-shock proteins (Hsps). We hypothesized that a heat-shock response modulates cytokine production in murine aortic vascular smooth muscle cells (VSMCs). VSMCs were exposed to 44 °C for 15–60 min, and subjected to interleukin-1β (IL-1β) or tumor necrosis factor α (TNFα), which induced interleukin-6 (IL-6) production. Expression of Hsps was examined with immunoblots, immunocytochemistry, or enzyme-linked immunosorbent assay (ELISA), and that of IL-6 with reverse transcription polymerase chain reaction (RT-PCR) or ELISA. Heat shock (44 °C for 45 min) induced Hsp72 in VSMCs at 4 h and elicited its maximal expression at 8 h after the end of heat shock. Treatment with IL-1β increased IL-6 transcription in VSMCs up to 24 h in an incubation time-dependent manner. Treatment with IL-1β or TNFα caused a concentration-dependent increase in IL-6 production in culture medium, which was attenuated by heat shock. Although treatment with Hsp72 or Hsp60 alone did not significantly affect basal IL-6 release into culture medium statistically, cotreatment with IL-1β and Hsp72, but not Hsp60 or boiled Hsp72, decreased IL-1β-induced IL-6 production in culture medium. Introduction of Hsp72, but not Hsp60, into VSMCs decreased IL-1β-induced IL-6 production in culture medium. These results indicate that the heat-shock response transcriptionally attenuated production of IL-6 in murine aortic VSMCs.

G1-027P
Hsp78 chaperone functions in restoration of mitochondrial network following extreme heat stress

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Under physiological conditions mitochondria of yeast *Saccharomyces cerevisiae* form a branched tubular network, the continuity of which is maintained by balanced membrane fusion and fission processes. Here, we show using mitochondrial matrix targeted green fluorescent protein that exposure of cells to extreme heat shock led to dramatic changes in mitochondrial morphology, as tubular network disintegrated into several fragmented vesicles. Interestingly, this fragmentation did not affect mitochondrial ability to maintain the membrane potential. Cells subjected to recovery at physiological temperature were able to restore the mitochondrial network, as long as an active matrix chaperone, Hsp78, was present. Deletion of HSP78 gene did not affect fragmentation of mitochondria upon heat stress, but significantly inhibited ability to restore mitochondrial network. Changes of mitochondrial morphology correlated with massive aggregation of mitochondrial proteins. On the other hand, the recovery of mitochondrial network correlated with disappearance of protein aggregates and reactivation of enzymatic activity of a model thermo-sensitive protein: mitochondrial DNA polymerase. Since protein disaggregation and refolding is mediated in mitochondrial matrix by Hsp78 chaperone collaborating with Hsp70 chaperone system, we postulate that effect of Hsp78 on mitochondrial morphology upon recovery after the heat shock is mediated by its ability to restore activity of unknown protein(s) responsible for maintenance of mitochondrial morphology.

G1-028P
Disruption of the gene for an Hsp70 cochaperone DjA1 results in impaired androgen receptor signaling and spermatogenesis

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Hsp70 family proteins are regulated by DnaJ/Hsp40 cochaperone proteins. DjA1 (dj2/HSDJ/hdj-2/rdj1) and DjA2 (dj3/rdj2) are major type I DnaJ proteins in mammalian cytosol. We have shown that DjA1 and DjA2 work similarly as a cochaperone of Hsp70 proteins in protein folding and mitochondrial protein import *in vitro*, and that these two DnaJs can complement each other in cultured cells (*J Cell Biol* 1997; *J Biol Chem* 2000). To study the possible differential roles of DjA1 and DjA2, we generated *DjA1*-deficient mice. Surprisingly, loss of *DjA1* in mice led to severe defects in spermatogenesis that involve aberrant androgen signaling. Transplantation experiments with green fluorescent protein-labeled spermatogonia into $DjA1^{-/-}$ mice revealed a primary defect of Sertoli cells in maintaining spermiogenesis at steps 8 and 9. In Sertoli cells of $DjA1^{-/-}$ mice, the androgen receptor markedly accumulated with enhanced transcription of several androgen-responsive genes, including *Pem* and *testin*. Disruption of Sertoli-germ cell adherents junctions was also evident in $DjA1^{-/-}$ mice. Experiments with $DjA1^{-/-}$ fibroblasts and primary Sertoli cells indicated aberrant androgen receptor signaling. These results revealed a critical role of DjA1 in spermiogenesis and suggest that DjA1 and DjA2 are not functionally equivalent *in vivo* [1].

Reference

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G1-029P
Complexes between GroEL and non-native proteins: their stoichiometry, conformation, stability and the role of ligands.

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Escherichia coli chaperone GroEL (hsp60) is known to prevent non-specific association of non-native proteins by the interaction with them and to promote their native structure formation in a ligand-dependent manner. In the present work, the complexes of GroEL with non-native proteins (lactalbumin, ribonuclease, lysozyme, casein, apocytochrome C and pepsin) were studied using size-exclusion and affinity chromatographies, small angle X-ray scattering, scanning microcalorimetry, limited proteolysis and fluorescence spectroscopy. The results demonstrate the following: (i) GroEL binds more then one protein target; (ii) the interaction with non-native protein increases GroEL thermostability and affects the orientation of its apical and intermediate domains as well as inter-ring distance; (iii) GroEL affinity to negatively charged non-native proteins is determinated by Mg^{2+} or Ca^{2+} ions; (iv) the interaction of GroEL with ADP, ATP and especially with co-chaperone GroES destabilize its complex with non-native protein; (v) GroEL affinity to non-native proteins is dependent on concentration of its ligands.

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G1-030P
Characterization of *Salmonella* FliS flagellar chaperone binding to flagellin

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Premature polymerization of flagellin (FliC) subunits, which are the main components of flagellar filaments is prevented by the FliS chaperones in the cytosol. Interaction of FliS with flagellin was characterized by isothermal titration calorimetry producing a dissociation constant of 5×10^{-8} M. Calorimetric measurements clearly demonstrated a binding stoichiometry of 1:1, in contrast to previous observations by gel filtration chromatography that a FliS dimer interacts with a FliC monomer. Binding became weaker at increasing temperature, but stable interaction persisted even at 55 °C, well above the denaturation temperature of flagellin. Binding experiments with terminally truncated fragments of flagellin confirmed that the C-terminal disordered region of flagellin is essential for FliS binding. The large negative entropy change indicated that significant ordering occurred upon FliS–FliC complex formation. Chaperone binding results in the stabilization of the disordered C-terminal region of flagellin into an α-helical conformation as suggested by CD measurements. Calorimetric experiments demonstrate that the central part of flagellin has a native-like, well folded conformation in the FliS–FliC complex indicating that FliS cannot keep flagellin in a partially unfolded export competent conformation in the cytoplasm.

G1-031P
Chaperone-like activity of alpha-chrystallin toward copper-induced aggregarion of aldose reductase

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Alpha-crystallin, the major protein component of the vertebrate eye lens, is a multimer of about 800 kDa, composed of two different subunits, alphaA and alphaB, both displaying sequence homology with small heat-shock proteins. Alpha-crystallin is known to interact with proteins undergoing denaturation, preventing loss of function and aggregation phenomena induced by different kinds of stress. Among chemical stress conditions, copper ion represents a relevant factor able to interfere with proper structures of proteins; this because its ability to induce site-specific oxidation processes and strongly interact with different functional groups, leading to protein precipitation. Bovine lens aldose reductase (ALR2) is a protein especially sensitive to copper ion. The oxidative modification of ALR2 induced by copper results in enzyme inactivation associated with the formation of an intramolecular disulfide bridge. When the molar ratio copper/ALR2 is >3, aggregation phenomena occur. Alpha-crystallin is able to protect ALR2 from both inactivation and aggregation induced by copper and this effect is dependent on the chaperone concentration. Calcium and magnesium, which are able to interfere with the chaperone activity of alpha-crystallin toward thermally stressed proteins, do not affect the ability of alpha-crystallin to protect ALR2 from the copper action. The protection exerted by alpha-crystallin toward copper-induced modification of ALR2 is at least in part associated to the ability of the chaperone to bind the metalion.

G1-032P
Small heat-shock proteins prevent thermally induced aggregation of actin filaments by formation of soluble complexes with denaturated actin

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The irreversible thermal denaturation of actin filaments (F-actin) is usually accompanied by aggregation. Temperature dependence of F-actin aggregation was measured by an increase in the light scattering at constant heating rate. It has been shown that F-actin aggregates within a narrow temperature range, from 55 to ~62 °C. There is a good correlation between the temperature dependence of aggregation and the thermal denaturation of F-actin measured by differential scanning calorimetry (DSC). Small heat-shock proteins (sHsp iV Hsp25/27 and α-crystallin) do not affect thermal denaturation of F-actin measured by DSC, but effectively prevent its heat-induced aggregation. Addition of sHsp results in shifting of the temperature of F-actin aggregation

from ~55–62 up to 80 °C. It has been shown by co-sedimentation experiments that sHsp do not bind F-actin at room temperature. However, thermally denatured F-actin heated up to 75 °C form soluble complexes with sHsp. Sedimentation coefficients of these complexes (estimated by analytical ultracentrifugation) and their size (determined by dynamic light scattering) were much less than the corresponding parameters of native or thermally denatured F-actin.

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G1-033P
Involvement of molecular chaperones in chloroplast protein import

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Plastids accomplish a great variety of metabolic functions in plants and eukaryotic algae. Although these organelles contain their own genomes, about 90% of chloroplast proteins are synthesized in the cytosol and post-translationally routed to their final destination. This process is carried out by a coordinate action of the chloroplast import machinery, molecular chaperones, and sequences present in precursor proteins, largely in the amino-terminal regions called transit peptides. We searched for *Escherichia coli* (DnaK) and endoplasmic reticulum (BiP) Hsp70 molecular chaperones binding sites, and plant Hsp70 interactions along the transit peptide of the ferredoxin-$NADP^+$ reductase precursor (preFNR). We determined that this transit peptide binds preferentially to CSS1, one of the Hsp70 isoforms present in the chloroplast stroma. Reduction of DnaK-binding tendency by mutagenesis in the transit peptide strongly affected the interaction with DnaK and CSS1. Surprisingly, the precursor with the lowest affinity for DnaK in its transit peptide was imported into chloroplasts with similar apparent K_m as the wild-type precursor and a twofold increase in V_{max}. The inclusion of chloroplast stroma during *in vitro* protein import experiments decreased the import rate of wild-type preFNR and did not affect the import of the mutant precursor. The reduction of BiP-binding tendency by mutagenesis only showed a slight decrease both in the interaction with CSS1 and in the rate of import of this mutant precursor. Thus, the interactions analyzed in this work seem not to be the main motor for protein import, suggesting that other factors may be responsible for unfolding and import of chloroplast precursors.

G1-034P
Plasmodium falciparum heat-shock protein 70 reverses thermosensitivity in an *Escherichia coli dnaK* mutant strain

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Heat-shock protein 70 (called DnaK in prokaryotes) is one of the most prominent group of chaperones whose role is to prevent and reverse protein misfolding. *Plasmodium falciparum* (Pf) is known to over-express heat-shock proteins including heat-shock protein 70 (Hsp70) during passage from the cold-blooded mosquito vector to the warm-blooded human host. Heat-shock protein 40 (called DnaJ in prokaryotes) whose function is to serve as a co-chaperone of Hsp70 has also been found to be stress inducible in *P. falciparum*. This suggests a potential cytoprotective function for these heat-shock proteins in the life cycle of the parasite. In this study, we investigated the *in vivo* chaperone role of PfHsp70 using *E. coli* cells with a deleted DnaK (*ÄdnaK52* strain) and cells whose DnaK function was compromised (*dnaK756* strain). Our results show that PfHsp70 has chaperone cytoprotective features as it was able to protect heat-sensitive *E. coli dnaK756* cells. *Escherichia coli* Ä*dnaK52* cells were not rescued from the heat stress possibly because they were not able to express PfHsp70. Using chimeric proteins, we investigated the possibility of interdomain communication between Hsp70 homologs from the two species. Whilst KPf, the chimera that had the ATPase domain of *E. coli* DnaK and PfHsp70 substrate-binding domain was able to reverse thermosensitivity, the reverse chimera PfK could not functionally substitute for DnaK in the *E. coli* cells. To further confirm that PfHsp70 can functionally substitute for DnaK, we intend to use another *E. coli* strain (*dnaK103*) with a truncated DnaK but whose DnaJ levels are normal as opposed to Ä*dnaK52* cells whose DnaJ levels are reduced. This is the first study that establishes an Hsp70 protein from *P. falciparum* with typical *in vivo* chaperone function.

G1-035P
Morphological analysis of the effect of bimoclomol against acute mercury-nephrotoxicity in rat

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A single exposure to mercuric chloride induces in rat acute nephrotoxicity, morphologically characterized by progressive necrosis of the straight portion of cortical proximal tubules and stimulates stress proteins at 24 h. Stress proteins are well-known molecular chaperones that function as lifeguards against proteotoxic stress. An excitant challenge for the research is the discovery of chaperone-inducing compounds and their clinical application. Bimoclomol (BM; Biorex R&D, Hungary) is a recently developed hydroxylamine derivative with cytoprotective effects, as stress-proteins coinducer, in cardiovascular disorders and diabetes. This *in vivo* morphological study was performed to shed light on the role of BM in mercury nephrotoxicity in rat. SD rats were divided into four experimental groups receiving: (i) mercuric chloride 1 mg/kg i.p.; (ii) BM 20 mg/kg per os; (iii) BM 6 h prior to mercury at the above doses; (iv) tap water per os as control. Tubular injury, nucleolar segregation and dense bodies were estimated on semithin sections at light microscopy. Ultrathin sections double-stained with uranyl acetate/lead citrate were observed under a Philips TEM CM10 at 80 kV. The renal distribution of stress proteins, such as metallothioneins, Hsp72, Hsp60, mt-Hsp70 was analysed by immunohistochemistry at different time after treatments. BM pre-treatment ameliorated ultrastructural tubular features, brush-border and membranes derangement and revealed almost normal mitochondria. At 14 and 24 h Hsp60 and mt-Hsp70 were overexpressed in proximal tubules treated with BM and mercury. BM attenuates mercury nephrotoxicity in rat. This might be due to its ability to enhance already preformed chaperones in the renal sites targets of mercury before the evidence of histological damage.

G1-036P
Crystallization and structure analysis of molecular chaperone-like reactivating factor for coenzyme B_{12}-dependent diol dehydratase

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Adenosylcobalamin (AdoCbl)-dependent diol dehydratase catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to the corresponding aldehydes. The enzyme undergoes mechanism-based inactivation by glycerol, a physiologic substrate. This inactivation is accompanied by the irreversible cleavage of the Co-C bond of enzyme-bound coenzyme and caused by the damaged cofactor that remains tightly bound to the apoenzyme. Rapid reactivation of the inactivated holoenzyme occurs in the presence of intact AdoCbl, ATP and Mg^{2+}. The diol dehydratase-reactivating factor (DDR), responsible for the reactivation, is composed of DdrA and DdrB and mediates the release of damaged cofactor from glycerol-inactivated holoenzyme. DDR is a new type of molecular chaperone that is involved in the reactivation of inactivated enzyme. In this study, the ADP-bound and nucleotide-free forms of DDR were crystallized, and the structures were solved by the MAD technique. Both forms of DDR exist as a dimer of αβ-heterodimer. The overall and domain structures of the α-subunit are essentially similar to those of the α-subunit of glycerol dehydratase-reactivating factor. The ADP molecule is bound in the ATPase domain of DDR in a similar manner to that in Hsc70. The linker domain connects the swiveling domain and the ATPase domain, and the insert domain bridges the gap between the ATPase domain and the neighboring β-subunit. The swiveling domain structurally connects the ATPase domain of the other α-subunit and the neighboring β-subunit. Based on the structures, the molecular mechanism of reactivation by DDR will be discussed.

G1-037P
Conformational changes and bound state conformation of maltose-binding protein upon interaction with the chaperone SecB: ESR and fluorescence studies

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SecB is a homotetrameric chaperone that forms part of the protein translocation machinery in *Escherichia coli*. We have previously investigated the bound state conformation of the model substrate bovine pancreatic trypsin inhibitor (BPTI) as well as the conformation of SecB itself by using proximity relationships based on site-directed spin labeling and pyrene fluorescence methods. The data suggested that SecB binds a collapsed coil of reduced unfolded BPTI, which then undergoes a structural rearrangement to a more extended state upon binding to SecB. In addition, ESR showed that also SecB undergoes a conformational change during this process. We have now studied the interaction of maltose-binding protein (MBP) with SecB by the same techniques.

G1-038P
Crystallization of the FaeE chaperone from *Escherichia coli* F4 fimbriae

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F4 fimbriae are expressed by enterotoxigenic *Escherichia coli* strains causing diarrhoea in neonatal or recently weaned piglets. F4 pili are thin, flexible structures consisting mainly of the major subunit FaeG, which also contains the adhesive properties. Mol and co-workers revealed that the F4 chaperone FaeE, involved in the biogenesis of F4 fimbriae via the FaeE/FaeD chaperone/usher pathway, is a homodimer and forms heterotrimeric complexes with the F4 fimbrial subunits. These results cannot be readily explained in the light of the current knowledge on the donor strand complementation/donor strand exchange mechanism. So far, only dimeric chaperone–subunit complexes have been identified. Chaperone dimerization has been observed for PapD and SfaE. In both cases the subunit-binding surfaces of these chaperones are involved in dimerization. Therefore, further research towards the F4 fimbrial biogenesis is required to clarify the molecular and structural basis of the F4 chaperone dimerization and the F4 chaperone–subunit interactions. Here, we report on the overexpression, purification and crystallization of the FaeE chaperone. The chaperone FaeE crystallizes in three crystal forms, all belonging to space group C2. Crystals of form 1 diffract to 2.3 Å and have unit cell parameters a = 195.7 Å, b = 78.5 Å, c = 184.6 Å, β = 102.2° . X-ray data for crystal form 2 were collected to 2.7 Å, using a SeMet variant of FaeE. The crystals have unit cell dimensions a = 136.4 Å, b = 75.7 = Å, c = 69.4 Å, β = 92.8°. Crystals of form 3 were formed in a solution containing the FaeE–FaeG complex and diffract to 2.8 Å. Unit cell dimensions are a = 109.7 Å, b = 78.6 Å, c = 87.8 Å, β = 96.4°.

G1-039P
The emerging role for small heat-shock proteins in the regulation of lipid composition and dynamics of cell membranes

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From previous data gained with cyanobacteria we inferred that under stress conditions the cellular pool of sHsps is divided into a cytoplasmic subfraction responsible for regular chaperone activity and a membraneous subfraction involved in membrane stabilization. In the present study, we show that specific lipid binding is not confined for cyanobacterial (Hsp17) and mammalian (alphaB-crystalline) sHsps, but also a feature of *Escherichia coli* sHsps IbpA and IbpB. The *E. coli* sHSP–membrane lipid interaction depends on the head group composition and the extent of lipid unsaturation and reveals specific differences for IbpA and IbpB. IbpA and IbpB can strongly regulate membrane fluidity and permeability as well. A comparative study conduced with wild type, $ibpAB^{-}$ disrupted and replacement strains ($ibpA^{+}$, $ibpB^{+}$, $ibpAB^{+}$) provides the first evidence for the involvement of sHsps in the homeostatic mechanisms controlling the fatty acid composition of membranes.

G1-040P
Hsp70 chaperone system actively remodels protein aggregates at the initial step of Hsp100-dependent reactivation

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Hsp100 heat-shock protein ClpB execute a crucial role in cellular thermotolerance in bacteria by cooperating with Hsp70 chaperone system (DnaK/DnaJ/GrpE) in resolubilization and reactivation of aggregated proteins. This cooperation widely broaden the ability of Hsp70 to renature even the large protein aggregates obtained by chemical or thermal denaturation. By developing an experimental model based on thermally denatured green fluorescent protein (GFP), we were able to collect real-time kinetic data of disaggregation reaction. Pre-incubation of aggregated GFP with Hsp70 system in the presence of ATP eliminates initial lag-phase of native GFP fluorescence increase observed when both systems are added simultaneously. This observation shows that Hsp70 interaction with aggregates is a rate-limiting step of reaction. Further analysis of Hsp70 action on aggregated substrates by glycerol-gradient centrifugation, light scattering measurements and GroEL trap mutant-binding experiments showed the change of biophysical and biochemical properties of aggregates. Results suggest that the initial step of Hsp100/Hsp70-dependent substrate reactivation is an active remodeling of aggregates by Hsp70 system.

G1-041P
The effect of Clp proteins on DnaK-dependent refolding of bacterial luciferases

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A study was made of the refolding of bacterial luciferases of *Vibrio fischeri*, *V. harveyi*, *Photobacterium phosphoreum*, and *Photorhabdus luminescens*. By reaction rate, luciferases were divided into two groups. The reaction rate constants of fast luciferases of *V. fischeri* and *Ph. phosphoreum* were about 10-fold higher than those of slow luciferases of *Ph. luminescens* and *V. harveyi*. The order of increasing luciferase thermostability was *Ph. phosphoreum*, *V. fischeri*, *V. harveyi*, and *Ph. luminescens*. The refolding of thermoinactivated luciferases completely depended on the active DnaK-DnaJ-GrpE chaperone system. Thermolabile fast luciferases of *V. fischeri* and *Ph. phosphoreum* showed highly efficient rapid refolding. Slower and less efficient refolding was characteristic of thermostable slow luciferases of *V. harveyi* and *Ph. luminescens*. Chaperones of the Clp family were tested for effect on the efficiency of DnaK-dependent refolding of bacterial luciferases in *Escherichia coli* cells. The rate and extent of refolding were considerably lower in the clpB mutant than in wild-type cells. In *E. coli* cells with mutant clpA, clpP, of clpX showed a substantially lower luciferase refolding after heat shock.

G2–Protein Structure and Stability

G2-001
Physics-based prediction of protein structure

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There are basically two different approaches to compute protein structure from a knowledge of the amino acid sequence. One is knowledge-based, i.e., it makes use of secondary-structure predictions, homology molding, threading, fragment coupling, etc., while the other makes use only of the potential energy describing the interactions among the atoms of the polypeptide chain, and between the chain and the surrounding solvent. The latter, physics-based approach, without use of knowledge-based information, can provide an understanding of how inter-atomic interactions dictate the final three-dimensional structure as a globally-optimized-potential-energy conformation. With an all-atom force field and an electrostatically-driven Monte Carlo (EDMC) search of the potential energy, it has been possible to compute the three-dimensional structures of proteins containing as many as 46 amino acid residues. To treat larger proteins, it has been necessary to use a hierarchical approach. In the first stage, the conformational space is searched extensively by using a simplified, physics-based united-residue (UNRES) representation of the polypeptide chain to locate the region of the global minimum of the all-atom potential energy. The UNRES conformations in this region are then converted to all-atom structures, and the search is then continued with the EDMC procedure, plus an implicit hydration model. Both the all-atom and hierarchical procedures will be described, and the results of these procedures, as judged by blind CASP tests, will be discussed.

G2-002
Ab initio simulations of protein folding pathways by molecular dynamics with the united-residue (UNRES) model of polypeptide chains

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Ab initio simulation of protein-folding pathways is currently impossible at the all-atom level even with the advantage of distributed computing. Reduced protein models used in this field are, on the other hand, arbitrary and can be used to study only general features of folding or are explicitly designed for specific proteins (the Go-like models). In this communication, we report the application of Langevin dynamics to the physics-based united-residue (UNRES) force field developed in our laboratory; this force field is sufficiently general to predict successfully the structures of real proteins based on the potential energy alone, as shown in the CASP experiments. Using the Lagrange formalism, we derived the equations of motion and modified the velocity Verlet algorithm to integrate them numerically. Test simulations were run on several proteins with sizes from 28 to 75 amino-acid residues and different structural classes, using our latest version of the UNRES force field parameterized by using our recently developed method for obtaining a hierarchical structure of the energy landscape. All alpha-helical proteins folded to the native-like structures, while there were fewer successful folding simulations for the alpha + beta and beta-proteins. For the last two classes of folds, mostly non-native alpha-helical structures were

obtained, although the native-like structures are lowest in energy. This can be attributed to neglecting the entropy factor in the current parameterization of UNRES, which was carried out using the decoy sets that constitute only low-lying local energy minima. Average folding times for successful folding simulations were of the order of nanoseconds, while even the ultrafast-folding proteins fold only in microseconds; this implies that the UNRES time scale is about three orders of magnitude larger than the experimental time scale because the fast motions of the secondary degrees of freedom are averaged out. Folding with Langevin dynamics required from 2 to 10 h of CPU time, on average, with a single AMD Athlon(tm) MP 2800+ processor depending on the size of the protein. With the advantage of parallel processing, this leads to the possibility to explore thousands of folding pathways and to predict not only the native structure, but also the folding scenario of a protein together with its quantitative kinetic and thermodynamic characteristics; this is shown using some examples. Converting the UNRES folding pathways into all-atom pathways and improvements of parameterization of the UNRES force field for MD simulations will also be discussed.

G2-003
Non-local interactions as a major component of the initial phase of a protein folding transition

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Do non-local or local interactions (long loops formation vs. secondary structure elements formation) dominate the critical initial phase of the folding transitions of globular proteins? Is the initial collapse a random condensation or programmed transitions? An answer to these questions was sought by determination of the kinetics of refolding of specifically labeled chain segments in a model protein, *E. Coli* adenylate kinase (AK), using time resolved fluorescence resonance energy transfer (FRET) detected stopped flow experiments (the "double kinetics" approach). The AK molecule consists of a single chain of 214 residues folded in three domains. The changes of the mean and the width of the distributions of the end-to-end distance of two secondary structure elements (helix, residues 169–188 and strand, residues 188–203) and two very long chain sections whose ends are in juxtaposition in the native structure (186 and 86 residues long) were determined in the denatured state, in the initial transient state, at 5 ms after initiation of refolding and in the native state. The double mixing stopped flow experiments showed that the ends of two very long segments, which were widely separated in the denatured state formed native like proximity within the dead time of the instrument (~5 ms). The short secondary structure elements folded with very three orders of magnitude slower kinetics. The end-to-end distance distributions of these segments were denatured like at the initial transient state and were folded only after 3 s. These results are compatible with the hypothesis that few very effective non-local interactions can be essential factor in stabilization of the early transient structures of folding of globular proteins and that the hydrophobic collapse of the molecule is probably not a random solvent exclusion process but rather a programmed transition.

G2-004
Functional roles for unfolded proteins

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The occurrence of unstructured regions of significant size (>50 residues) is surprisingly common in functional proteins. As methods for exploring the functions of proteins (and other macromolecules) within cells and in more complex *in vitro* systems have arisen, it has become clear that unfolded and partly-folded proteins have important roles to play in numerous cellular processes and signaling events. The extent and variety of the role of such proteins has not been determined as yet, but promises to provide a fruitful new field for thinking about the molecular mechanisms of biological processes. Many gene sequences in eukaryotic genomes encode entire proteins or large segments of proteins that lack a well-structured three-dimensional fold. Disordered regions can be highly conserved between species in both composition and sequence and, contrary to the traditional view that protein function equates with a stable three-dimensional structure, disordered regions are often functional, in ways that we are only beginning to discover. Many disordered segments fold on binding to their biological targets (coupled folding and binding), whereas others constitute flexible linkers that have a role in the assembly of macromolecular arrays. Spectroscopic methods such as NMR have now advanced in sensitivity and resolution, to the point at which the structural propensities and dynamics of sizeable disordered proteins in solution can be thoroughly characterized. Possible reasons for the inclusion of an unfolded state in the normal functioning of a protein will be discussed, including the need for mutual refolding to form intimately associated complexes, the requirement for reversibility of binding and the thermodynamic consequences of structure formation *in situ*.

G2-005
The pairwise energy content estimated from amino acid composition discriminates between folded and intrinsically unstructured proteins

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Instrinsically unstructured/disordered proteins (IUPs) exist in highly flexible, unfolded structural state largely devoid of well defined secondary and tertiary structure elements. Although these proteins are predicted to be common in living organisms, the existing datasets are limited in size and heterogeneous in terms of the type of disorder they cover. In this work we propose a simple model for the physical basis of protein disorder. The underlying assumption is that intrinsically unstructured proteins adopt no stable structure because their amino acid composition does not allow sufficient favorable interactions to form. Globular proteins, in contrast, are composed of amino acids which have the potential to form a large number of favorable interactions. On this ground, the polypeptides encoding globular and disordered proteins can be distinguished. Our method is based on the estimation of the total pair-wise interaction energy using a quadratic form in the amino acid composition of the protein only. This approach is validated by the good correlation of the estimated and actual energies of proteins of known structure. Applying this method to a dataset of disordered proteins, their predicted energy values were clearly shifted towards less favorable energies compared to globular proteins. As the novel algorithm has not been trained on unstructured proteins, it substantiates the concept of protein disorder, i.e. that the lack of well-defined 3D structure of many proteins and protein domains is the consequence of their specific amino acid sequence. The algorithm can be turned into a position-specific scoring scheme, and it predicts ordered and long disordered regions of proteins with high accuracy.

G2-006
Prediction of protein flexibility from geometrical constraints

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Protein function is strongly coupled to the conformational flexibility. Molecular Dynamics (MD) simulations are routinely used to study protein dynamics. However, sampling of the conformational space by MD-simulations is computationally expensive and therefore limited. CONCOORD attempts to alleviate this obstacle by predicting protein flexibility based on geometrical constraints from a given structure and searching for alternative conformations that fulfill these constraints. The results obtained from CONCOORD simulations depend on the choice of atomic parameters like Van-der-Waals radii and the definition of other geometrical constraints (hydrogen bonds, salt bridges, etc.). Particularly the hydrogen bond network is important for the conformational flexibility of a protein, therefore the probability of opening of a hydrogen bond is estimated by determining its hydrophobic protection. We strive to extract the parameters used in CONCOORD from experimental data. High resolution X-ray structures from the Protein Data Bank are used for the calibration of Van-der-Waals-radii whereas X-ray structures of proteins of which more than one structure is available are used to estimate other parameters.

G2-007P
Structural and functional characterization of a novel carboxypeptidase inhibitor from ticks

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A novel proteinaceous metallo-carboxypeptidase inhibitor, named tick carboxypeptidase inhibitor (TCI), was isolated from the blood-sucking tick *Rhipicephalus bursa*. The full-length cDNA of TCI contains an open reading frame coding for a protein of 97 residues that consists of a hydrophobic signal sequence preceding mature TCI, a 75-residue cysteine-rich protein (12 Cys). The deduced amino acid sequence of TCI shows no homology to other known proteins; the C-terminus, however, resembles those of other protein metallo-carboxypeptidase inhibitors. Recombinant TCI expressed in *Escherichia coli* is fully functional and inhibits carboxypeptidases of the A/B subfamily with equilibrium dissociation constants in the nanomolar range. Structural analyses by CD and NMR indicate that TCI is a protein strongly constrained by disulfide bonds, unusually stable over a wide pH range and highly resistant to denaturing conditions. The three-dimensional crystal structures of recombinant TCI bound to either bovine carboxypeptidase A or human carboxypeptidase B were determined and refined at 1.7 and 2.0 Å resolution, respectively. The structure of TCI defines a protein motif consisting of two domains that are structurally equivalent despite their low sequence homology, each one with a short α-helix followed by a small twisted antiparallel β-sheet. TCI domains anchor on the surface of mammalian carboxypeptidases in a novel double-headed manner not previously described for carboxypeptidase inhibitors. As a tight binding inhibitor of plasma carboxypeptidase B, also known as TAFI, recombinant TCI enhances fibrinolysis *in vitro* and may have potential for applications to prevent or treat thrombotic disorders. Therefore, the structures of these new complexes may be valuable to improve the biomedical application of TCI as a thrombolytic drug.

G2-008P
Introduction of a new disulfide bridge into a cold-active enzyme reduced activity while heat-stability is increased

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Disulfide bonds are found in some extracellular proteins. They may confer extra stability upon those proteins and promote folding into the catalytically active conformation. Such cross-linking may, however, concomitantly affect the catalytic function of enzymes by restricting dynamic movement within the conformational ensemble. Cold-adaptation is necessary in many organisms and must involve alteration of their proteins at the genetic level. Loosening of interatomic attractive forces by amino acid selection is a major evolutionary target in such enzymes in order to allow sufficient dynamic movement at low temperatures. This is often accompanied by a reduction in heat-stability, although local effects may suffice to ensure catalytic efficiency without sacrificing global stability. A single cysteine is present in a cold-adapted alkaline phosphatase from a marine *Vibrio* bacterium. The position of this cysteine only two residues away from the nucleophilic serine in the active-site cleft makes it a good candidate for exploring the effect of disulfide bridges on enzyme activity. We have exchanged five serine residues in the immediate vicinity to the native cysteine one by one with a cysteine using site-directed mutagenesis and find that a new disulfide bridge is formed in one instance. As predicted by current theories, this had the effect of increasing heat-stability as well as reducing the catalytic activity. Similar effects have been obtained by cross-linking with glutaraldehyde.

G2-009P
Cloning, bacterial expression, purification and structural characterization of N-terminal domain of γ-Gliadin

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The gene encoding N-terminal half of gamma-Gliadin from wheat endosperm has been subcloned into a thioredoxin expression system (pET102/D-Topo) and over-expressed as fusion protein in *E. coli*. Thioredoxin was removed by enterokinase cleavage and by acid cleavage at the respective engineered recognition sites. The highly soluble protein was purified to homogeneity by affinity and reverse phase chromathography. The secondary structure of the purified protein domain was analysed by Circular Dichroism, showing an spectral shape common to a Poly(Pro) II conformation. The spectrum is dominated by a large negative peak centered around 201 nm, and a broad shoulder centered around 225 nm. Also the temperature denaturation process was studied. The differences observed in the spectra show two main tendencies, the increment of the shoulder intensity, and the drop of the intensity

of the peak around 201. When the sample was cooled down, the change on intensity of the shoulder around 225 was completely reversible. On the contrary, it was found that the reversibility on the change of the CD signal around the 201 nm peak was addressed up to a 90%. To our knowledge this is the first report of the cloning, and expression of recombinant repetitive domain of reserve proteins from wheat. This has important implications for the characterization, of these sort of proteins.

G2-010P
NMR structure of the fifth and sixth transmembrane segments of the mitochondrial oxoglutarate carrier

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The structures of the fifth and sixth transmembrane segments of the bovine mitochondrial oxoglutarate carrier (OGC) and of the hydrophilic loop that connects them were studied by CD and NMR spectroscopies. Peptides F215-R246, W279-K305 and P257-L278 were synthesized and structurally characterized. CD data showed that at high concentrations of TFE and SDS all peptides assume α-helical structures. 1H-NMR spectra of the three peptides in TFE/water were fully assigned and the secondary structures of the peptides were obtained from nuclear Overhauser effects, 3JαH-NH coupling constants and αH, chemical shifts. The three-dimensional solution structures of the peptides were generated by distance geometry calculations. A well-defined α-helix was found in the region L220-V243 of peptide F215-R246 (TMS-V), in the region P284-M303 of peptide W279-K305 (TMS-VI) and in the region N261-F275 of peptide P257-L278 (hydrophilic loop). The helix L220-V243 exhibited a sharp kink at P239, while a little bend around P291 was observed in the helical region P284-M303. Fluorescence studies performed on peptide W279-K305, alone and together with other transmembrane segments of OGC, showed that the W279 fluorescence was quenched upon addition of peptide F215-R246, but not of peptides K21-K46, R78-R108 and P117-A149 suggesting an interaction between TMS-V and TMS-VI of OGC.

G2-011P
Tertiary structure of water-soluble fragment of cytochrome b5 is influenced by negatively charged membranes at pH 7.2

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Water-soluble fragment of cytochrome b5 (Cyt b5) with the noncovalently bound heme is responsible for the functional redox properties of cytochrome b5, which is connected to the membrane by its C-terminus. The conformational state of Cyt b5 has been studied at pH 7.2 in the presence of negatively charged phospholipid membranes. Model membranes were prepared from negatively charged lipids (POPG, DPPG), or as a mixture with neutral phospholipids by sonication. The diameter of these vesicles is 300–400 Å. The molar ratio of phospholipid/Cyt b5 changed from 25:1 to 500:1. A variety of physical methods such as near – and far – UV circular dichroism, tryptophan fluorescence and the heme absorbance, differential scanning microcalorimetry and FPLC were used in the studies of Cyt b5 structure. It was shown that the tertiary structure of all Cyt b5 molecules is affected by the presence of negatively charged vesicles only at a high molar ratio, 500:1 at pH 7.2, but its helical secondary structure is preserved. The native structure of Cyt b5 in the presence of vesicles undergoes the transition to an intermediate state, similar to its molten globule in aqueous solution. In this intermediate state Cyt b5 interacts with the membrane surface. At a low molar ratio POPG/Cyt b5, such as 25:1, 50:1, the tertiary structure of unbound Cyt b5 resembles that of the native one. The denaturing effect of mixed phospholipid vesicles is even more pronounced. It is suggested that Cyt b5 structure might be affected by membrane surface to facilitate its interactions with redox-partners.

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G2-012P
Snake hemoglobins tetramer stability analyzed by osmotic stress, gel filtration, SAXS and modeling

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A series of articles published from 1987 to 1999 [1, 2] reported evidences of a dimer–tetramer transition mechanism in some snake hemoglobins. According to this hypothesis, hemoglobins of these species, when in the deoxygenated state would predominate as tetramers. Nevertheless, when oxygenated, these proteins would split into dimers within the Red Blood Cells, having a physiological advantage. According to Matsuura et al. (1989) [2] this dissociation should occur due to substituted residues (Glu 43 → Thr and Glu 101 → Val) in the alpha1-beta2 interface. The lack of these two negative charges should favor the dissociation into dimers. The aim of our work was to analyze hemoglobins of some snake species (*Crotalus durissus terrificus*, *Helicops modestus*, *Boa constrictor* and *Liophis miliaris*) in order to perform functional characterization and to determine the level of dissociation of those proteins, in order to confirm the existence or not of the dissociation mechanism linked to oxygenation. Analytical gel filtration chromatography, used for the determination of the dimer–tetramer association constant 4K2, showed that snake hemoglobins analyzed in this work are stable tetrameric forms in solution. SAXS analysis, showed radius of gyration and maximum dimension very similar to finding to human hemoglobin. The analyzed snake hemoglobins showed cooperative oxygen binding, in the whole tested pH range, both in the presence as in the absence of ATP. The data gathered using osmotic stress showed that deoxy-hemoglobins from *Crotalus* and *Helicops*, in the absence of anions, assume a conformational state, denominated P or To, as proposed by Colombo & Seixas (1999) [3] for human and bovine hemoglobin, and described more recently also for other hemoglobins [4, 5]. Due to the lack of evidences supporting the dissociation hypothesis, we performed a molecular modeling of *L. miliaris* hemoglobin, in order to analyze the dimeric interface of this protein to understand the real effect of the residues substitution and the lack of dissociation. We carried out a comparative structural analysis between modeled hemoglobin from *L. miliaris* and fish, chicken and human hemoglobins. The results showed that the beta chain residues Glu 43 and Glu 101 are not essential for the maintenance of the tetrameric form. Some chicken and fish hemoglobins possess these residues substituted, not being reported as dimeric hemoglobins. In conclusion, we did

not find evidences to support the proposed mechanism of dissociation for *L. miliaris* hemoglobin, nor other snake hemoglobins.

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G2-013P
Effects of substitutions of conserved non-functional residues by Ala on the folding of apomyoglobin

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To understand the folding mechanism of proteins that goes through the formation of several intermediate states, it is necessary to study of equilibrium and kinetic intermediates as well as the transition states formed on the protein folding pathway. Six residues in myoglobins are well-conserved, though being unconnected with the primary oxygen-carrying function of these proteins. A role for these conserved, non-functional residues in the folding pathway of apomyoglobin has been suggested. In order to test this hypothesis, urea-induced equilibrium and kinetic unfolding/refolding reactions of mutant apomyoglobins with substitutions of conserved non-functional residues by Ala have been studied by tryptophan fluorescence. The properties of these mutant proteins have been compared with those of wild-type apomyoglobin. All of them have a compact globular structure, but are much destabilized in comparison with the wild-type protein. The folding pathways of the mutants are similar to that of wild-type and include the formation of a folding kinetic intermediate state. In contrast to wild type protein, for which the equilibrium intermediate population was estimated to be 10%, for mutant proteins it is 40–100%. The chevron plot for the transition between native and intermediate states has been constructed for all mutants, giving a possibility to estimate φ-values for a rate-limiting barrier, which is about 0.3 for all mutant proteins.

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G2-014P
Substitution of negatively charged Glu445 by uncharged Ala precludes complete two-electron reduction of the heme d/heme b595 binuclear oxygen-reducing site in cytochrome bd oxidase from *Escherichia coli*

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Cytochrome bd is a terminal respiratory oxidase required by bacteria for survival and growth in microaerophilic environments (Baughn & Malamy. *Nature* 2004; **427**: 441–444). The enzyme does not pump protons, however oxidation of ubiquinol by O_2 catalyzed by cytochrome bd is linked to generation of membrane potential (Jasaitis et al. *Biochemistry* 2000; **39**: 13800–13809). Wild type and Glu445Ala mutant cytochrome bd oxidases from *Escherichia coli* were studied by time-resolved electrometric and spectrophotometric methods. High spin heme b_{595}, a component of the binuclear O_2 reducing site, appears to be not missing from the mutant as originally proposed (Zhang et al. *Biochemistry* 2001: **40**: 8548–8556), but remains ferric even in the presence of dithionite. Although both the wild type and mutant oxidases can react with O_2, the rate of formation of the oxoferryl species in the wild type is >2 orders faster than that in the mutant. In contrast, no difference between these two enzymes is observed during generation of membrane potential associated with electron 'backflow' reactions following the photolysis of CO bound to ferrous heme d in the one-electron reduced enzyme. Heme b_{595} is a major contributor to electron redistribution from heme d after CO photolysis of the one-electron reduced enzyme, however electron transfer between hemes d and b_{595} appears to be non-electrogenic and therefore is not accompanied by net proton uptake or release. In contrast, electron transfer to heme b_{558} is electrogenic. Glu445 is one of two protonatable groups which uptake protons upon reduction of the di-heme site composed of hemes b_{595} and d. The E445A mutation prevents complete two-electron reduction of the binuclear site.

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G2-015P
Tryptophan fluorescence as a probe of acyl carrier protein conformation

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Acyl carrier protein (ACP) is a small flexible protein that must undergo reversible conformational change as it delivers thioester-linked fatty acyl substrates to over two dozen enzymes involved in the synthesis bacterial lipids, endotoxins, and other products. We have used site-directed mutagenesis to introduce tryptophan residues as local fluorescent probes of *Vibrio harveyi* ACP, which lacks endogenous Trp and is largely unfolded at neutral pH. Addition of millimolar Mg^{2+} to all ACP mutants resulted in a folded helical conformation as assessed by circular dichroism (CD). Consistent with the NMR structure of ACP, Trp residues at positions 46, 50 and 72 adopted a more non-polar environment (i.e. fluorescence emission maxima shifted from >350 nm to Mg^{2+}-induced folding, whereas residues 25 and 45 remained in a polar environment. Covalent attachment of myristic acid also stabilized the folded conformation of all ACP mutants by CD and native gel electrophoresis analysis, but had much less effect on the environmental polarity of Trp residues at positions 46, 50 and 72. Our data indicate that two mechanisms thought to similarly stabilize the native folded conformation of ACP (fatty acylation and divalent cation binding) actually produce distinct differences in the environments of specific residues. These results may be relevant to the mechanisms by which different enzymes can recognize and interact with acyl-ACPs.

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G2-016P
Insight into the structure–function relation of ostreolysin, a pore-forming protein from the edible mushroom *Pleurotus ostreatus*

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Aegerolysin family is a novel protein family with increasing number of isolated or predicted proteins from evolutionarily distant organisms. Its members are proposed to be involved in bacterial sporulation, fungal fruiting and virulence. A representative of this family, ostreolysin (Oly) is a cytolytic protein isolated from the fruiting bodies of the edible oyster mushroom (*Pleurotus ostreatus*). Its pore-forming activity is essentially dependent on the presence of liquid-ordered lipid domains, such as lipid rafts. Here we report the complete nucleotide sequence of Oly, together with the structural, thermodynamical, and biological characterization of its conformational states at different pH values, ionic strength, and temperatures. Utilizing different spectroscopic methods, PAGE electrophoresis and hemolytic activity assays, we have demonstrated that, in a narrow pH range around neutral and at room temperature, native Oly is monomeric, with rigid tertiary and predominantly β-sheet secondary structure, and hemolytically active. It appears that the protonation/deprotonation equilibria of two Cys and/or two His residues are involved in the regulation of hemolytic activity and stability of Oly between pH 7 and 8. Between pH 2 and 3, the protein is irreversibly converted into a partially unfolded state which binds ANS, and exhibits disrupted tertiary structure and enhanced, nonnative α-helical structure. At alkaline pH, Oly undergoes two irreversible base-induced transitions, characterized by disruption of tertiary and most secondary structure, resulting in a loss of hemolytic activity. Our results provide an insight into the role of pH in structural alterations of ostreolysin, which can be relevant to environmental modulation of its lytic activity.

G2-017P
Dimer–tetramer transition and the allosteric regulation of phosphofructokinase-2 from *E. coli*

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Binding of MgATP to the negative allosteric site of Pfk-2 promotes a dimer–tetramer oligomeric transition, but a dimeric inhibited intermediary has not yet been isolated. The inactive tetramer can be reverted to the active dimer upon increasing the fructose-6-P concentration. In this work we study the effect of two mutations that affect the allosteric behavior in order to understand the inhibition mechanism of the enzyme: Y23D and C295F. GdnHCl denaturation studies of the normal enzyme in the presence of MgATP by size exclusion chromatography, dynamic light scattering and intrinsic fluorescent measurements do not show the presence of any dimeric species, suggesting a strong thermodynamic coupling between dimer association upon MgATP binding. The Y23D mutant is not inhibited by MgATP and does not change its aggregation state in the same range of MgATP concentrations used for the normal enzyme, although the MgATP-induced tetramer formation of this mutant can occur at high protein concentrations. The C295F mutant exhibits an apparently higher tetramer stability compared to the normal enzyme, as indicated by GdnHCl-induced unfolding experiments, which in turn results in a higher fructose-6-P concentration necessary to revert the inactive tetramer to the active dimer, compared to the normal enzyme. Taken together these results indicate that MgATP binding and dimer association are strongly coupled processes, and that tetramer formation is a crucial factor for the modulation of the allosteric inhibition of Pfk-2.

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G2-018P
Differential enantioselectivity in methoxychlor O-demethylation by CYP2C enzymes

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Methoxychlor, a currently used pesticide undergoes metabolism by cytochrome P450 enzymes forming a chiral mono-phenolic derivative [1,1,1-trichloro-2-(4-hydroxyphenyl)-2'-(4-methoxyphenyl) ethane; Mono-OH-M] as main metabolite. Human CYP2C8, CYP2C9 and CYP2C19 enzymes were previously shown to possess high enantioselectivity in methoxychlor metabolism favoring the formation of S-Mono-OH-M. In the current study, other members of the CYP2C family were examined for chiral preference in Mono-OH-M formation. Purified rabbit CYP2C3 showed no enantioselectivity in Mono-OH-M formation, whereas purified CYP2C5 slightly favored the formation of R-Mono-OH-M in reconstituted systems. Furthermore, the possible causes for the differential enantioselectivity in metabolism were investigated with molecular modeling. Two differences were observed in the amino acid sequence of the active center of CYP2C3 enzyme as compared to CYP2C9 active center, namely Val102Leu and Phe103Ala. Docking studies showed that the change in position 103 might be the cause for the absence of enantioselectivity in methoxychlor metabolism by CYP2C3.

G2-019P
A comparison of the aggregation of different CFTR-derived peptides, the N-terminal huntingtin fragments and different fusion proteins encompassing the aggregation-prone sequences of CFTR and/or huntingtin

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The best characterized amino acid sequence known to induce protein aggregation is the polyQ tract, responsible for the aggregation of huntingtin and several ataxins. Recently, the ag region in CFTR (cystic fibrosis transmembrane conductance regulator) has been found to induce the aggregation of the CFTR-derived C-terminal peptides. This region corresponds to the so-called switch motif, a region important for the function of several bacterial ABC transporters where it is required for the steps subsequent to ATP binding and hydrolysis. However, the function of this motif in the CFTR, the only ion channel among the ABC transporters, remains unknown. The aim of this study was to compare the aggregation of different CFTR-derived peptides, the N-terminal huntingtin fragments (with 75 or 18 glutamine

repeats) and different fusion proteins encompassing the aggregation-prone sequences of CFTR and/or huntingtin. The fusion resulted in altered distribution of aggregates. For example, no nuclear inclusions, typical for huntingtin aggregation, were observed. Fusion had also a significant impact on aggregates' morphology. In many cases protein was accumulated in the form of honeycomb. Cotransfection experiments are being currently performed to investigate aggregation specificity determinants in analyzed constructs. The specificity of aggregation process is a topic of special importance as sequestration hypothesis infers functional deficit of crucial cellular proteins being 'trapped' in a protein aggregate.

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G2-020P
Unfolding and aggregation of β-lactoglobulin: isolation and structural characterization of stable intermediates

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The present study focuses on the characterization of stable molecular species that occur during heat-induced unfolding and aggregation of β-lactoglobulin (β-Lg). The first step of this process involves the exposition of the native free sulfhydryl group of β-Lg (Cys 121) that is involved in subsequent intermolecular sulfhydryl/disulfide exchange reactions. According to the experimental conditions, we were able to isolate a disulfide-linked dimer and a stable monomer with a non-native sulfhydryl exposed on β-Lg surface (Cys119). The dimerization reaction was favored by the presence of copper which catalyzed the oxidation of β-Lg free sulfydryl exposed groups. The rate of dimer formation increased with the concentration of copper. The copper-dependent conversion rate of native protein into covalently linked-dimer was found to be 7 to 10-folds faster for B variant than for A variant (variants A and B differ by two amino-acids substitution). The formation of non-native β-Lg monomer was a concomitant event that occurred during β-Lg unfolding throughout intramolecular sulfhydryl/disulfide exchange reaction. This stable non-native monomer exhibits some structural characteristics of a molten-globule. The results reported here complete our knowledge on the unfolding/aggregation mechanism of β-Lg.

G2-021P
A structural and functional characterization of Tom70: a receptor of the mitochondrial import machinery

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Some 99% of mitochondria's protein complement is encoded by the eukaryotic organism's nuclear genome, and imported into the organelle post-translationally with the co-operation of various cytosolic chaperones such as MSF or Hsp70. The TOM Complex (Translocase of the Outer Membrane) is a multi-subunit complex residing on mitochondrial outer membrane. The translocase consists of several receptors (Tom70, Tom20 and Tom22); the pore forming Tom40, and several other smaller subunits. Proteins destined for the mitochondrion's inner membrane, such as carrier family proteins, are usually targeted to the organelle via multiple internal targeting sequences present in the precursor's primary structure. The receptor Tom70 recognizes this class of chaperone-bound precursor proteins and facilitates its translocation through the TOM complex's import pore into the intermembrane space, where its further localization is facilitated by the TIM22 complex. To better understand Tom70-mediated carrier import we recently performed a biophysical characterization of the receptor: the first such study for a component of the mitochondrial import machinery. We demonstrated, via analytical ultra-centrifugation, that Tom70 exists as a highly asymmetric monomer, incapable of dimerising autonomously. Unfolding studies, as measured by circular dichroism and native tryptophan fluorescence, show that the protein unfolds via a multi-step pathway and undergoes major conformational change at physiological temperatures. This suggests that Tom70 has discrete, differentially folding domains. Like other TPR-containing proteins, Tom70's inherent fluidity might suggest a coupled binding/folding mechanism for client protein recognition. Through further experiments, such as stopped-flow kinetics, tryptophan scanning, and our on-going crystallographic analyses, we hope to gain further insight into the organization of the Tom70's multi-domain structure and how its binding mechanism actuates its role in the import of carrier proteins.

G2-022P
Change on the vibrations upon ligand binding to a protein

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Protein–ligand association has been assumed to be dominated by factors such as the hydrophobic effect, hydrogen bonding, electrostatic and van der Waals interactions. However there is an additional mechanism, the vibrational effect, which might play an important role due to increased flexibility in the protein–ligand complex. This concept has been introduced by Sheraga et al. in early 1960s but it has been lucking the experimental evidence. From the experimental side here we present the results obtained by neutron scattering on dihydrofolate reductase upon binding methotrexate. The vibrational density of states shows a significant difference between the bound and unbound species at low frequency modes of the spectra leading to a softening upon binding. From the theoretical side molecular dynamics and normal mode calculations were carried out to understand the molecular basis of this binding mechanism based on computer simulations. Normal mode calculations show the best agreement with the experimental results. Simulation results show that different protein conformations exhibit slightly different vibrational density of states especially in the low frequency region (<20/cm), which leads to a broadening of this range of the spectra. It became clear that the low frequency range of protein vibrations, which is important in biological functioning, is inhomogeneous broadened because of the existence of different conformational substates.

G2-023P
Interfacial properties of sunflower storage proteins

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The secondary structure and the interfacial behavior of 2S albumin fraction of sunflower have been investigated at an oil-water interface. The aim of the study was to characterize the sunflower seed storage proteins in relation to potential applications as emulsifiers in food systems and to their reported allergenicity for humans. The 2S albumin fraction of sunflower was separated by HPLC and fractions corresponding to SF-LTP, SFA8 and three further mixtures of albumins (called fractions A, B and C) were collected. The surface tension and surface rheology of the proteins were measured before and after heat treatment. Heat did not affect the surface properties of SF-LTP and SFA8, but did change the surface elasticity of the other three albumin fractions, resulting in greater surface activity. The effect of heat on the secondary structure of the proteins was also studied by circular dichroism spectroscopy, which agrees with the surface activity data. The emulsification properties of the proteins were studied by determining the droplet size of the emulsions formed. The results showed that albumin fraction A formed the smallest droplets and was therefore the best emulsifier amongst the proteins of the 2S albumin fraction, whilst SFA8 was the most surface active at an oil–water interface. SF-LTP showed neither good emulsification properties nor high surface activity. However, the fact that heating had no effect on its secondary structure is consistent with its reported allergenicity.

G2-024P
Structural-based differential stability in the YoeB-YefM toxin–antitoxin system

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Although natively unfolded proteins are being observed increasingly, their physiological role is poorly understood. One case in which a natively unfolded state of a protein appears to have physiological significance is that of the YefM antitoxin protein of the *Escherichia coli* YoeB-YefM toxin–antitoxin (TA) module. An imperative element in the proper functioning of TA systems is the poor metabolic stability that the antitoxin demonstrate compared to it toxin partner. The YefM antitoxin is a natively unfolded protein, lacking secondary structure even at low temperatures. In contrast, its toxin partner, YoeB, demonstrated well-folded conformation at physiological temperatures with reversible unfolding up to a temperature of 56 °C. We have further shown that YefM and YoeB interact and form tight complex in solution. We suggested that the structural-based differential stability between the two system components serves as a critical element in the proper function of the TA module, as low structural stability expose the antitoxin protein to cellular proteases, hence granting it with lower physiological stability than its toxin partner. Toxin–antitoxin modules attracted much interest in the last few years due to their potential use as antibacterial targets. We chose to address this challenge by designing peptides capable of interfering with the YefM-YoeB interaction, thus releasing the toxin to execute its detrimental function. To that end, using peptide array technology, we have identified a short, 9 aa-long, linear determinant within the YefM protein that is involved in YoeB interaction, which would be later used as structural basis for the design of such peptide agents.

G2-025P
Cloning, expression and characterization of superoxide dismutase from *Deinococcus radiodurans*

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Deinococcus radiodurans is well-known for its remarkable resistance to ionizing radiation. It has been a target for intensive studies on the mechanism of repair of DNA double-strand breaks. On the other hand, its protein components are also valuable sources for structure–function relationship studies due to their possible unique structures which may confer special properties, such as thermostability or radioresistance. We have cloned, expressed and characterized a manganese superoxide dismutase (MnSOD) from *D. radiodurans* (DeiRa). DeiRa MnSOD with a His-tag expressed in *E. coli* was purified to homogeneity on SDS-PAGE; its molecular mass was determined by mass spectrometry to be 24 883 Da (theoretical mass 24 876 Da). The enzyme specific activity was assayed spectrophotometrically as 824 U/mg. Thermostability of DeiRa MnSOD was assayed by pre-treatment of enzymes at temperatures from 40 to 100 °C followed by assay of residual enzyme activity. This enzyme was found to be stable at 90 °C for 20 min without loss of activity. At 100 °C it partially precipitated and lost about half of enzyme activity. As a comparison, MnSOD from *E. coli* exhibited a higher specific activity of 5263 U/mg and a much poorer thermostability; it was heat-denatured at 60 °C with a loss of 70% of activity. We also assayed the radioresistance of DeiRa MnSOD by exposure to X-ray irradiation at a total dose of 2.6 kGy at 4 °C. followed by assay of residual enzyme activity. The DeiRa MnSOD was partially inactivated after X-ray irradiation. The extent of inactivation was found to be dependent on the protein concentration.

G2-026P
Fibril formation and aggregation of a model protein can proceed from different structural conformations of the partially unfolded state

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Protein misfolding and aggregation are interconnected processes involved in several neurodegenerative and systemic diseases. Mutations or changes in the environmental conditions can partially unfold the native state of a protein, causing it to aggregate into well defined fibrillar structures. A lot of interest has been directed to the features of the metastable partially unfolded state that precedes the aggregated state. In our work, human muscle acylphosphatase (AcP) has been destabilized under different conditions using urea, high temperature or high pH as destabilizing agents. With the addition of different amounts of 2,2,2, trifluoroethanol ranging from 5 to 25% (v/v), we were able to obtain partially unfolded states with distinct structural conformations. We show herein that AcP can form fibrillar and non-fibrillar aggregates with a high β-sheet content from very differently structured

partially unfolded states. Moreover, we demonstrate that a partially unfolded state rich in α-helical structure is not a prerequisite for the aggregation of AcP. The absence of aggregation under some of the conditions used here seems to be attributable to the conditions themselves rather than to the structural features of the partially unfolded state populated in these media. Taken together our results support the conclusion that fibril formation and aggregation of AcP are independent from just one specific structure of the partially unfolded state and occur when the solution conditions are favorable enough to form stable hydrogen bonds.

G2-027P
SDSL studies of an ankyrin-sensitive lipid-binding site in erythroid β-spectrin

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Spectrin, a high molecular weight flexible rod-like protein formed by head-to-head association of two heterodimers composed of α (280 kD) and β (247 kD) subunits, is a major component of membrane skeleton in human erythrocyte. It was recently shown that the region within β-spectrin responsible for attachment of the membrane skeleton via ankyrin to integral membrane proteins includes also a lipid-binding site which displayed sensitivity to ankyrin inhibition. We have studied this region by making a series of double spin-labeled peptides and analyzing their dipolar interaction by paramagnetic resonance method. Our results indicate that the ankyrin-sensitive lipid-binding site of β-spectrin exhibit an α-helical conformation, which is in agreement with our molecular modeling experiments. The investigations are being proceeded to elucidate the whole structure of the above mentioned region and its changes during interactions with lipids and/or ankyrin.

G2-028P
De novo proteins based on albebetin and functional peptide from alfa2-interferon and factor HLDF: design, biological and fibrillogenic properties

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Based on the *de novo* protein albebetin (ABB) and its variant albeferon (ABBI), including a human alfa2-interferon (alfa2-IFN) octapeptide LKEKKYSP (130–137), we obtained three functional ABB variants, which ensure antiviral and differentiating activities. *De novo* protein ABB-df was engineered by including the biologically active hexapeptide TGENHR (41–46) from factor HLDF of human leukemia cell line HL-60 into the N-terminus of ABB. Study of the chimerical protein showed that it induced differentiation and inhibited proliferation of the HL-60 cells almost as efficiently as the hexapeptide. A peptide LKDRHDF (30–36) of alfa-IFNs was inserted into α- and N-terminus of ABBI to obtain proteins possessing antiviral activity. Both proteins demonstrate antiviral activity, the protein with N-terminal location of both fragments being almost as active as alfa2-IFN. Structural studies of all chimerical proteins showed that it maintained regular secondary structure of ABB and conceivably they are in molten globule state under physiological conditions as well as ABB. Recently was shown that molten globule state is an important step of the proteins fibrillation process. In that way the *de novo* proteins are a good model for fibrillation study. We examined the fibril formation of three *de novo* proteins: ABB and its derivatives ABBI and ABB-df. Obtained data showed that all three proteins are able to rapid fibril formation. The atomic force microscopy analyses revealing the morphological polymorphism of fibrils in ABB, ABBI and ABB-df solutions. ABBI and ABB-df formed fibrils more rapidly, then ABB. We suggest that peptides may compensate negative charges of ABB and promote protein's self-assemblage to fibrillar aggregates, thus electrostatic interaction stabilizes the amyloid structures.

G2-029P
Structural studies of NOTCH ligands JAG1 and DLL3

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The NOTCH signaling pathway has a central role in a wide variety of developmental processes and it is not therefore surprising that mutations in components of this pathway can cause dramatic human genetic disorders. Our work is aimed at determining the structure of regions of interest in the NOTCH ligands JAG1 and DLL3. We first focused on the extra-cellular region of human JAG1, which is predicted to contain 16 epidermal growth factor-like (EGF) repeats. The G274D mutation in the EGF-2 of JAG1 is associated with a familial form of tetralogy of Fallot, a severe heart malformation. Interestingly, we found that a peptide spanning the C-terminal part of EGF-1 and the complete EGF-2 repeat, which corresponds to the entire exon 6 of the Jag-1 gene, represents an autonomously folding unit. Our current work is focused on the structure determination, by NMR and/or X-ray crystallography, of regions of interest in the extra-cellular region of human DLL3, which contains 6 EGF-like repeats. A missense mutation (G385D) in the EGF-4 has been associated with axial skeletal defects characteristic of spondylocostal dysostosis (SD) syndrome. A synthetic gene of a selected target of DLL3, spanning a region comprising EGF-3 to 5, was designed with optimized codon usage for over-expression in *E. coli* and assembled by PCR from synthetic oligonucleotides. As a second target, a fragment spanning the whole extra-cellular region of DLL3 was amplified from human DLL3 c-DNA and cloned into a suitable vector for expression in *P. pastoris*. Expression trials are currently underway.

G2-030P
Hydrostatic pressure and osmotic stress as non-genomic factors leading to polymorphism in amyloidogenic self-assembly of proteins

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Self-assembly of proteins may be conceptualized a conformational struggle of polypeptide chains to reduce frustration of

hydrogen bonding, and burry hydrophobic side chains. We are showing that changes in hydration of polylysine and insulin lead to either radical increase of the propensity to aggregate [1], or to a new conformational variant of fibrils [2]. Such ''alien'' forms are kinetically stable, even under conditions favoring ambient fibrils. When used as templates for seeding daughter generations of amyloid, the fibrils reproduce its stacking patterns regardless of environmental biases. This parallels the ''prion strains'' phenomenon – hinting at its possible generic character [2]. Amyloid polymorphism may be also explored under high-pressure conditions, which disfavor loosely packed structures. While moderately high pressure reduces efficiency of the insulin aggregation [3], it simultaneously favors a unique circular amyloid [4]. That the fibrils' growth under high-pressure conditions is accompanied by a highly repetitive bending to one side conveys an argument for anisotropic distribution of void volumes within the ambient fibrils. Polymorphism of insulin amyloids may be explored by changing physical conditions affecting the protein hydration. This provides new clues as to the mechanisms of aggregation and widens its possible bio- and nanotechnological applications.

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G2-031P
A study of the interactions of green tea polyphenols with Angiogenin and Ribonuclease A

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Green tea polyphenols are known to inhibit angiogenesis, the process that involves the growth of new blood vessels. Angiogenesis provides essential nourishment for the growth and metastasis of tumors. Angiogenin, one of the most potent blood vessel inducers known plays a significant role in this process. Angiogenin is a unique member of the ribonuclease superfamily known to have both ribonucleolytic and angiogenic activities. Translocation of angiogenin to the nucleolus is an essential step in the mechanism by which angiogenesis is induced by the protein. Angiogenin is homologous to Ribonuclease A (RNase A). The ribonucleolytic active site of RNase A comprises residues His12, Lys41 and His119, the corresponding residues in angiogenin are His13, Lys40 and His114. In addition to the ribonucleolytic site, angiogenin has a cell binding site and a nuclear translocation site. Previously, we have investigated the effect of green tea polyphenols, mainly the catechins, on an angiogenin-like protein induced angiogenesis process (Maiti et al. *Biochem Biophys Res Comm* 2003; **308**: 64–67). We have also looked at the kinetics of the interactions of green tea polyphenols and its major component (-) epigallocatechin gallate with Ribonuclease A (Ghosh et al. *Biochem Biophys Res Comm* 2004; **325**: 807–811). In this report, we substantiate our experimental findings by docking studies. Each of the major components of green tea has been docked to angiogenin and RNase A. Results indicate that the preferred location for binding of the green tea polyphenols is the nuclear translocation site of angiogenin, as speculated earlier whereas for RNase A, a disruption in the base binding site is implicated.

G2-032P
A preliminary study of the stability, catalytic activity and Zn affinity of human carboxypeptidase M (CPM) and some of its glycosylation mutants

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Human carboxypeptidase M (CPM) is a membrane-bound metallo-carboxypeptidase with one catalytic zinc ion bound per active center. It is attached via a glycosylphosphatidylinositol anchor to the plasma membrane. In various body fluids though, including amniotic fluid, seminal plasma and urine, CPM is found in a soluble form. CPM was overexpressed in a soluble form in the yeast *Pichia pastoris* to produce the recombinant CPM wild type (rCPMwt) in quantities amenable to the study, stability, catalytic activity and Zn affinity. rCPMwt was treated with N-glycosidase F, an enzyme that removes N-linked glycans from protein substrates. SDS-PAGE analysis of samples treated for various times revealed that all four potential N-linked glycosylation sites were utilized. To determine the specific role of the Asn-Xaa-Ser/Thr N-glycosylation sites, some sites were eliminated by site-directed mutagenesis of Asn to Gln. The stability of the rCPMwt and the glycosylation mutants was measured by guanidine-induced denaturation at pH 7.2. The denaturation curves are very similar, suggesting a minimal effect of the glycosylation on the stability of CPM. Direct measurement of Zn by using a spectrometric assay with the metallochromic indicator 4-(2-pyridylazo)resorcinol showed that, at room temperature, Zn is easily extracted from the denatured but not from the native form of rCPMwt. Zn binding had important consequences for the folding/unfolding equilibrium. Mutation of at least one of the glycosylation sites had a significant effect on the catalytic parameters of the fluorescent substrate dansyl-Ala-Arg (k_{cat} and K_m).

G2-033P
Functional studies of metallothionein gene of *Triticum durum* at molecular level

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Metallothioneins are small, cystein rich, low molecular weight polypeptides found in almost all organisms. They are thought to be involved in heavy-metal detoxification and metabolism of essential trace elements. Unlike their mammalian conuterparts, plant MTs have not been characterized in terms of cellular regulation and function. A novel gene sequence, with two exons and one intron, encoding a metallothionein was identified in durum wheat *Triticum durum*. The mRNA of the protein was isolated, and sequence alignment analysis on both cDNA and translated protein showed that *T. durum* MT (dMT) can be classified as a Type 1 MT with three C-X-C motifs in each of the N- and C-terminal domains. dMT was overexpressed in *E. coli* as a fusion protein (GSTdMT). GSTdMT was purified intact with Cd by using affinity and size exclusion chromatography. The MT protein forms two metal binding domains bridged with long hinge region. This long hinge region is conserved among distinct plant species with some identical amino acids. Homology modeling was used to predict a 3D structure for dMT. Conservation of the sequence of the hinge region may indicate involvement of such MTs in mechanisms other than metal detoxification. The experimental observations and structural modeling also suggest an

alternative role which may involve interactions with DNA. The predicted structure for the hinge region shows similarity with DNA binding proteins. Functional implications of the predicted structure are being investigated through *in vivo* and *in vitro* DNA-protein interaction assays including; whole genome PCR-based screening, chromatin immunoprecipitation assay (ChIP), ChIP-ChIP. Results will be discussed in the general framework of functional features of different types of plant metallothioneins.

G2-034P
Structural analysis of DNA topoisomerase I of *Leishmania donovani*

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Leishmania donovani, the causative organism for visceral leishmaniasis, contains an unique heterodimeric DNA-topoisomerase IB (TOPO I). The catalytically active enzyme is constituted of a large subunit (LdTOPIA), which contains the non-conserved N-terminus and a phylogenetically conserved core domain, while the small subunit (LdTOPIB) harbors the C-terminus region with a characteristic tyrosine residue in the active site; the linker domain is unlocated in neither subunits. Heterologous co-expression of LdTOPIA and LdTOPIB in a topoisomerase I deficient yeast strain, produces extracts with significant DNA relaxation activity. The present work determines the non-essential domains using a serial delections approach of both subunits and its posterior combination, and the results show a dispensable C-terminal region in LdTOPIA with 61amino acids length, whereas the LdTOPIB is able to relax the supercoiled DNA with a reduced extension of 87 amino acids from the 261 initials. Combination of both deletions results in an active dimeric protein, as described before in the human topoisomerase I. Mutational analysis of key amino acids involved in relaxation activity, including the Tyr 222 identified as the active site and the four basic amino acids surrounding it in the crystallographic structure. We created three additional mutants of leishmanial TOPO I with different camptothecin sensitivity, testing their resistance to potential leishmanicidal drugs, by different *in vivo* assays.

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G2-035P
Influence of hydrophilic lecithins on the heat-induced interaction between whey-proteins and casein micelles

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Heat treatment of milk (70–90 °C) causes denaturation of whey-proteins, that may either lead to the aggregation of these denatured proteins or the whey-protein coating of the casein micelles. This research focuses on the effect of heat on the properties of milk proteins (casein and whey-proteins) and their heat-induced interactions in different concentrations of whey-proteins and different types and concentrations of lecithins, by using the following techniques. PCS (Photon Correlation Spectroscopy) It was shown that the increase of whey-proteins and heating time increased the casein micellar size due to complexation with whey-proteins, starting at 80 °C/5min, when the ratio between casein and whey-proteins was 4 and 2. Moreover, only hydrophilic (hydroxylated or hydrolysed) lecithins had a decreasing increase on the casein micelle diameter after the heat treatment, in the presence of whey-proteins. Centrifugal Photosedimentometry: It was concluded that the increased diameter observed by PCS was not only due to whey protein deposition onto the casein micelles, but was also partly due to the consequent aggregation of the whey-protein coated casein micelles.

DSC (Differential Scanning Calorimetry): The whey-protein denaturation temperature was shifted upward upon addition of hydrolysed lecithin, which indicated a heat-stabilizing effect of hydrolysed lecithin on whey-proteins. From these combined data, it was concluded that the heat-induced interaction between casein micelles and whey-proteins could be reduced by addition of hydrophilic lecithins, which, according to our opinion, is due to the fact that the latter stabilizes the unfolded state of the denatured whey-proteins and hence minimizes its aggregation and interaction with other proteins.

G2-036P
Amyloid in atherosclerotic plaques: an important player in a severe disease

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Amyloid is insoluble aggregated proteins deposited in the extra cellular space and is commonly found in many tissues in association with aging. These deposits were over-looked for long time and believed to be of no importance. However, elucidation of the nature of some deposits has generated a radical change in the general conception of amyloid deposits. Some of these are now strongly believed to be important actors in the pathogenesis of certain age-related diseases, including Alzheimer's disease and type-2 diabetes. Age-related localized amyloid is common in vascular tissues. Aortic intimal amyloid is seen in close association with calcified areas of atherosclerotic plaques and we postulate that it may play a yet unknown role in the pathogenesis of atherosclerosis. To further explore that possibility, knowledge about the protein nature of this amyloid is crucial. The aim of the present study is therefore to characterize the main protein in the amyloid of atherosclerotic plaques, elucidate its pathogenesis and its effects in the vessel wall. In an autopsy investigation, we have collected material from aortic atherosclerotic lesions in 55 patients. The amyloid was seen in 47% of the well-developed atherosclerotic lesions with necrosis and calcification. We use a proteomic approach in order to identify the amyloidogenic protein and today we have several interesting protein candidates that we are evaluating by immunohistochemistry and Western blot.

G2-037P
Kinetic properties of some mutants of L-pyruvate kinase

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Activity of L-pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40) is regulated by phosphorylation of the serine residue in the peptide sequence …Arg(9)-Arg(10)-Ala-Ser(12)-Val(13)…, located in the regulatory domain of the protein molecule. In this report we have studied the effect of point mutations,

made in positions 9, 10 and 13 flanking this phosphorylation site, on catalytic properties and stability of the enzyme. Ala, Lys or Glu were introduced into each of these positions as described elsewhere. The mutants were specially selected to alter the charge situation of the regulatory domain, and check the hypothesis whether redistribution or introduction of new ionic groups in the regulatory domain can mimic the regulatory effect of phosphorylation of the Ser(12) residue. We have found that in some of these three positions both catalytic activity and enzyme stability can be affected by the mutations. However, the cooperative behavior of the substrate reaction cannot be induced by replacement of the amino acids, but needs phosphorylation of the Ser(12) residue.

G2-038P
Molecular dynamics studies of protein and peptide misfolding

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Amyloid beta peptides spontaneously aggregate and build senile plaques and fibular tangles in the brains of Alzheimer disease patients. The polypeptide has been the subject of extensive *in vitro* and computational research. Still, the pathway to aggregational forms and their exact conformations remain largely unclear. Here, we present an extensive Molecular Dynamics (MD) approach simulating the peptide in various temperatures, pH conditions and with different charge states of the N- and C-termini, thus exploring the conformational space of the peptide at large. Our results demonstrate the stability of various conformations including beta hairpin structures in modified conditions, suggesting that all of these conformations are allowed in one condition but usually are not sampled by MD simulations since they might be separated by high free energy barriers.

G2-039P
Multiple mapping method: a new approach to improve sequence to structure alignments

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A major bottleneck in comparative protein structure modeling is the quality of input alignment. Although a number of alignment methods are available, none of them produce consistently good alignments for all cases. Even for a single alignment method, the different combinations of the choice of mutation matrix and gap penalty parameters produce alignments that are better in some cases and worse in others. To overcome this problem, we have developed a new approach, called Multiple Mapping Method (MMM) that optimally combines alternative alignments obtained from different methods or from different choices of mutation matrix and gap penalty parameters. The MMM algorithm first identifies the variable regions from the consensus alignment of the set of input alignments. The alternative variable regions are scored using a composite scoring function, which determines its fitness within the environment defined by the template structure. The best scoring variable regions from the set of alternative segments are combined with the core part of the alignment to produce the final MMM alignment. The application of this method to a benchmark database of 6500 protein pairs shows that the average accuracy of the MMM alignments are consistently better than the average input alignments.

G2-040P
Heterologous overexpression and characterization of a novel amine-synthesizing enzyme, *N*-substituted formamide deformylase

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Background: *N*-Substituted foramamide deformylase (NfdA) from *Arthrobacter pascens* F164 is a novel deformylase involved in the metabolism of isonitriles [1]. The enzyme catalyzes the deformylation of an *N*-substituted formamide, which is produced from the corresponding isonitrile. There is currently a great deal of interest in the catalytic mechanism of this enzyme. A more detailed understanding of it will be obtained by analysis of its three-dimensional structure. However the yield of the enzyme obtained from the wild strain was too low to perform such analyses. Here we report the heterologous overexpression of *A. pascens* F164 NfdA in an active form by using a strong expression system for *Streptomyces*, and the purification and characterization of the recombinant NfdA.

Results: The *nfdA* gene encoding NfdA enzyme was cloned into different types of expression vectors for *Escherichia coli* and *Streptomyces* strains. Expression in *E. coli* resulted in the accumulation of an insoluble protein. However, *Streptomyces* strains transformed with a P*nitA*-NitR system, which we very recently developed as a regulatory gene expression system for streptomycetes [2], allowed the heterologous overproduction of NfdA in an active form. When *S. lividans* TK24 transformed with pSH19-*nfdA* was cultured under the optimum conditions, the NfdA activity of the cell-free extract amounted to 8.5 U/mg, which was 29-fold higher than that of *A. pascens* F164. The enzyme also comprised about 20% of the total extractable cellular protein. The recombinant enzyme was purified to homogeneity and characterized.

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G2-041P
A transient syntaxin/SNAP-25 interaction serves as ready-available binding site for synaptobrevin

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The SNARE proteins syntaxin 1a, SNAP-25, and synaptobrevin 2 play a central role during Ca^{2+}-dependent exocytosis at the nerve terminal. While syntaxin and SNAP-25 are located in the plasma membrane, synaptobrevin resides in the membrane of neurotransmitter-loaded synaptic vesicles. It is thought that gradual assembly of these proteins into a membrane-bridging trans-SNARE complex initiates exocytosis. According to this model, syntaxin and SNAP-25 constitute an acceptor complex for the vesicular synaptobrevin. *In vitro*, however, syntaxin and SNAP-25 form a stable complex that contains two syntaxin molecules, one of which is obstructing the binding site of synaptobrevin. To elucidate the assembly pathway of the synaptic SNARE proteins in more detail, a variety of biophysical methods were applied. During complex formation major structural changes from mostly unstructured monomers to a tight α-helical complex occur. I found that these transitions are accompanied by large favorable binding enthalpies. Furthermore, kinetic studies revealed that

assembly begins with the slow and rate-limiting interaction of syntaxin and SNAP-25. For their productive interaction, their SNARE helices must come together simultaneously. Interestingly, when the interaction of syntaxin and SNAP-25 was blocked, binding of synaptobrevin was hindered. This suggests that binding of synaptobrevin occurs after the establishment of the syntaxin-SNAP-25 interaction. Moreover, binding of synaptobrevin was inhibited by an excess of syntaxin, suggesting that a transient 1:1 interaction of syntaxin and SNAP-25 serves as the ready available on-pathway SNARE assembly intermediate.

G2-042P
Recognition of intrinsically unstructured proteins: the ''facile-binding'' model

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Intrinsically unstructured proteins (IUPs) fulfill essential biological functions such as signaling, regulation or chaperons that require fast, specific and yet reversible binding. This type of recognition is dependent on the exceptional adoptability IUPs: they are devoid of regular tertiary fold in solution and their binding to the partner is coupled to disorder to order transition. To explain their effective functioning we reasoned that IUPs display limited local structure in solution that presage their bound conformational state. A high correlation between the intrinsic secondary structure preferences of IUPs and their complexed structures was demonstrated. The prediction accuracy of IUPs exceeded that of the random sequences and was comparable to the template molecules, indicating their folding autonomy. Hence we propose a modular architecture for IUP structures: segments of considerable stability are connected by flexible linkers. The locally ordered regions can feature as preformed structural elements that can play distinguished role in recognition. In our interpretation these motifs present a complementary interface for the partner and thus could serve as initial contact points, the binding of which facilitates the reeling of the flexible regions onto the template. This finding implies that intrinsically unstructured proteins draw a functional advantage from preformed structural elements, as they enable their facile, kinetically and energetically less demanding, interaction with their physiological partner.

G2-043P
Structural analysis and discrimination of outer membrane proteins

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Discriminating the outer membrane proteins (OMPs) from genomic sequences and location of membrane spanning β-strands in OMPs are challenging problems in Bioinformatics. We have developed several datasets, such as, well annotated OMP and α-helical membrane proteins sequences, non-redundant datasets of OMP sequences and structures, and globular proteins belonging to four different structural classes. Using these datasets, we have systematically analyzed the characteristic features of the 20 amino acid residues in globular and OMPs. We found that the residues, Glu, His, Ile, Cys, Gln, Asn and Ser show a significant difference between the amino acid compositions of globular and outer membrane proteins. The higher occurrence of Ser, Asn and Gln in OMPs than globular proteins might be due to their importance in the formation of β-barrel structures in the membrane, stability of binding pockets and the function of OMPs. The surrounding hydrophobicity of non-polar amino acid residues in globular proteins is significantly higher than that of OMPs. We have devised a statistical method for discriminating OMPs from other globular and membrane proteins. Our approach correctly picked up the OMPs with an accuracy of 89% for the training set of 337 proteins. On the other hand, our method has correctly excluded the globular proteins at an accuracy of 79% in a non-redundant dataset of 674 proteins. Furthermore, the present method is able to correctly exclude α-helical membrane proteins up to an accuracy of 80%. We have developed a web interface for discriminating OMPs and predicting the membrane spanning segments in OMPs. It is freely available at http://psfs.cbrc.jp/tmbeta-net/.

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G2-044P
Active site mutants of *Vibrio* sp. alkaline phosphatase

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Psychrophilic organisms produce enzymes that are efficiently able to perform their catalysis at temperatures where enzymes from mesophilic or thermophilic species are generally unable to sustain viable metabolism. Increased catalytic efficiency at low temperatures as well as increased thermolability are common features of these enzymes and are believed to be a consequence of enhanced structural flexibility. Exactly how this flexibility is brought about is not fully understood and can be achieved with different strategies in different enzyme families. Cold-active alkaline phosphatase (AP) from a *Vibrio* sp. is very thermolabile. It is still unknown weather these characteristics are originated in the active site locus or in more general overall structural factors. Three metal ions are present in the active site (usually Zn, Zn, Mg). Homology alignment shows that the amino acid residues that bind the two zinc ions are totally conserved in all known APs. However, two residues that bind the Mg ion are different in various APs: Asp153/Lys328 in *E. coli* AP, are His116/Trp274 in *Vibrio* AP, and His/His in mammalian APs. This may explain their different catalytic efficiencies. We have mutated Trp274 in *Vibrio* AP to Lys, His or Ala. These various mutants all displayed increased heat stability, together with reduced substrate affinity (K_m) and lower overall reaction rate (k_{cat}). These results are consistent with the general theory that catalytic rate and substrate binding are closely related to structural flexibility. In the presence of a phosphate acceptor (1M Tris or diethanolamine) the enzyme exhibited transphosphorylation activity. Under those conditions the replacement of Trp274 by Lys shifted the pH optimum towards lower pH. The exact pH optimum of the Lys mutant was decided by the pK_a of the buffer ion that acts as a phosphate acceptor. At conditions where the enzyme-bound intermediate was hydrolyzed, the pH optimum did not change. Overall, the results show that a single amino acid substitution in the active site is sufficient to alter the structural stability as well as kinetic properties of the *Vibrio* AP.

G2-045P
Molecular properties, mutagenesis and overexpression of the lipase from *Pseudomonas fragi*: a case study for cold-activity

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Low temperature-active enzymes have recently received increasing attention because of their relevance for both basic and applied research. In biotechnology, novel opportunities might be offered by their catalytic activity at low temperature and, in some cases, unusual specificity [1]. A large number of researchers, fascinated by the realm of cold-adaptation, is aiming to a deeper understanding of its basis. In this context, we have recently cloned and overexpressed a lipase from the psychrophilic bacterium *Pseudomonas fragi* (PFL) that we are employing as a tool to study the molecular bases of cold-activity, thermolability and tendency to aggregation upon overexpression. Despite a high degree of similarity with other *Pseudomonas* lipases, PFL displays unique properties in term of thermolability and substrate specificity [2]. In this work, well known methods like site directed mutagenesis have been joined by evolutionary and biophysics approaches to widen our knowledge. Mutagenesis studies suggested an important role of the so-called "lid", a mobile surface structure that modulates access to the active site in both specificity and thermostability. As the cold-active lipase is expressed in *E. coli* mainly as insoluble aggregates, Fourier transform infrared spectroscopy (FT-IR) applied to producing cells disclosed the kinetics of aggregation of the recombinant protein when expression conditions were modulated.

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G2-046P
Expression of hepatitis C virus (HCV) helicase domains and investigation of possible inhibition mechanism

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Hepatitis C virus (HCV), the member of the Flaviviridae family, is a human pathogen that represents a serious epidemiological threat. The non-structural protein 3 (NS3) of HCV is one of the most promising targets for anti-HCV therapy because of its multiple enzymatic activities such as RNA-stimulated nucleoside triphosphatase (NTP-ase), RNA helicase and serine protease. The helicase activity is indispensable for viral replication *in vivo*, presumably unwinding double-stranded replication intermediates allowing RNA amplification. The NS3 helicase (C-terminal part of NS3) is composed of three domains, and two of them – domain 1 and 2 – form the most probable site of ssRNA binding, thus constituting a good target for enzyme inhibition. For NMR studies of helicase-inhibitors complexes we cloned and overexpressed the NS3 helicase and separately its domains 1 and 2. The helicase and domain 2 were cloned using a baculovirus system in insect cell culture. Domain 1 was cloned in a bifunctional vector by means of homologous recombination in yeast and overexpressed in *E. coli*. CD studies of domain 1 were carried out to determine the stability and secondary structure of the protein. Preliminary 1D NMR experiments revealed that domain 1 is involved in interaction with several inhibitors that interacted with the entire helicase but not with its domain 2. Further NMR experiments (1H-15N HSQC) with two helicase inhibitors 1-(5,6-dichlorobenzotriazole)-β-D-ribofuranose (DCBTR) and NS3 peptide inhibitor allowed the identification of putatively specific binding sites.

G2-047P
The effect of osmolytes on the activity and stability of mushroom tyrosinase

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Tyrosinase is a copper containing enzyme that catalyzes the o-hydroxylation of phenolic compounds (cresolase activity) and successive oxidation of them to the corresponding o-quinones (catecholase activity). The thermodynamical stability and remained activity of mushroom tyrosinase from *Agaricus bisporus* in 10 mM phosphate buffer, pH 6.8, stored at two temperatures of 4 and 40 °C were investigated in the presence of three different amino acids (His, Phe and Asp) and also trehalose as osmolytes for comprising with the results obtained in the absence of any additive. The kinetics of inactivation was found to obey the first order law and the inactivation rate constant (k_{inact}) value is the best parameter describing the effect of osmolytes on the kinetic stability of the enzyme. Trehalose and His have the smallest value of k_{inact} (7×10^{-5}/s) in comparison with their absence (2.5×10^{-4}/s). Moreover, to obtain the effect of these four osmolytes on the thermodynamical stability of the enzyme, protein denaturation by dodecyl trimethylammonium bromide (DTAB), as a cationic surfactant, was investigated. The sigmoidal denaturation curves were analyzed according to the two states model of Pace theory to find the Gibbs free energy change of the denaturation process in aqueous solution at room temperature (ΔG_{H2O}), as a very good thermodynamic parameter indicating the chemical stability of the protein. Experiments were carried out in the absence and in the presence of any osmolytes. From denaturation curves values 16.4, 20.4, 21.0, 23.7 and 31.4 kJ/mol obtained for ΔG_{H2O} in the absence and presence of trehalose, His, Phe and Asp, respectively. Hence using of above osmolytes is a proper strategy to increase the stability of tyrosinase.

G2-048P
High resolution hydration analysis as a tool for the label free characterization of protein folding and binding events

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The vital biological functions of proteins in the cell are based upon their intact native three dimensional structure. Stabilized by water, the structure of proteins is very much dependent on an equilibrium with its environment (hydration state) which can be

significantly influenced by external factors such as temperature, ionic strength or drugs. Disturbance of this sensitive equilibrium often leads to protein misfolding and aggregation, a common pathological mechanism of degenerative disorders of aging including Alzheimer's Disease, Parkinson Disease and prion disorders. The recent development of molecular acoustics opened up new application possibilities including high resolution monitoring of hydration changes in proteins. Here we demonstrate the sensitivity of the molecular acoustic measurements by detecting the binding of various ligands to proteins. Application examples include the binding of small molecules ligands to soluble. Molecular acoustic measurements enabled a quantitative detection of binding and allowed the determination of binding constants in absence of labeling. We then monitored protein folding and mis-folding events since mis-folding is emerging as common mechanism for important neurodegenerative diseases of aging i.e. Alzheimer' disease and prion disorders. Thermal denaturation and renaturation of several proteins were characterized using temperature scans and revealed significant differences in general folding behavior, the number of folding intermediates, general thermal stability and renaturation efficiency. Thus, molecular acoustics prove to be an efficient analytical approach to characterize the structural behavior, stability and binding of various proteins under native conditions. We therefore believe that this enabling technologies will soon open up new possibilities for analytical research from laboratory to clinical applications.

G2-049P
Structural studies of the glucose-dependent insulinotropic polypeptide (GIP) by NMR and molecular modeling

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Type-2 diabetes mellitus is characterized by a decreased responsiveness of peripheral tissues to insulin and a diminished and delayed pancreatic beta-cell response to glucose. Therefore, novel therapeutic agents that normalize the beta-cell response to glucose are of considerable interest in the treatment of type-2 diabetes. One candidate agent is the gastrointestinal peptide, gastric inhibitory polypeptide (GIP), which acts as a major insulin-releasing hormone through the enteroinsular axis. The present study is designed to thoroughly investigate the solution structures of GIP and its analogues and to predict their tertiary structure using a molecular modeling approach. Use of NMR spectroscopy is vital to determine the three-dimensional structure of the peptide in solution-state. Therefore, to understand the basic structural requirements for the biological activity of GIP, the solution structure of the major bio-active fragment, GIP(1-30)amide, and the native GIP(1-42) were investigated in 2,2,2-trifluoroethanol (TFE-d3)-water solvent mixture, by proton NMR spectroscopy. The molecular modeling structures were characterized by an alpha-helical conformation between residues F6-A28 and residues F6-K32 for GIP(1-30)amide and GIP(1-42) respectively. Preliminary NMR studies of native GIP(1-42) in pure water suggest the formation of an alpha-helical confirmation between residues Y10-K30. This structural information will be used to examine the structure–activity relationship studies of GIP receptor agonist/antagonists. This will help advance the field of therapeutic peptide drug development with application in treatment of type-2 diabetes.

G2-050P
Solid-state MAS NMR of the outer-membrane protein G from *Escherichia coli*

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Uniformly 13C-, 15N-labelled outer-membrane protein G (OmpG) was expressed in *E. coli* for structural studies by magic-angle spinning (MAS) solid-state NMR. Inclusion bodies of the recombinant, labeled protein were dissolved and purified under denaturing conditions, and the protein was refolded in detergent. OmpG was then reconstituted into lipid bilayers to obtain large quantities of two-dimensional (2D) crystals. The crystallized samples were used for structure investigation by solid-state cross-polarization magic-angle spinning (CP/MAS) NMR. Despite the large size of the protein (33 kDa), signal patterns for several amino acids, including threonines, prolines and serines were resolved and identified in 2D proton-driven spin-diffusion (PDSD) spectra of the uniformly labeled sample.

G2-051P
The substitution of intrinsic tryptophans allows to probe local rearrangements upon unfolding of goat α-lactalbumin

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In order to test the occurrence of local particularities during the unfolding of Ca^{2+}-loaded goat α-lactalbumin (GLA) we replaced Trp60 and -118, either one or both, by Phe. In contrast with alternative studies, our recombinant α-lactalbumins are expressed in *Pichia pastoris* and do not contain the extra N-terminal methionine. The substitution of Trp60 leads to a reduction of the global stability. The effect of the Trp118Phe substitution on the conformation and stability of the mutant, however, is negligible. Comparison of the fluorescence spectra of these mutants makes clear that Trp60 and -118 are strongly quenched in the native state. They both contribute to the quenching of Trp26 and -104 emission. By the interplay of these quenching effects, the fluorescence intensity changes upon thermal unfolding of the mutants behave very differently. This results in a discrepancy of the apparent transition temperatures derived from the shift of the emission maxima ($T_{m,Fl\ \lambda}$) and those derived from DSC ($T_{m,DSC}$). However, the transition temperatures derived from fluorescence intensity ($T_{m,Fl\ int}$) and from DSC ($T_{m,DSC}$), respectively, are quite similar and thus, no local rearrangements are observed upon heat-induced unfolding. At room temperature, the occurrence of specific local rearrangements upon GdnHCl-induced denaturation of the different mutants is deduced from the apparent free energies of their transition state obtained from stopped-flow fluorescence measurements. By Φ-value analysis it appears that, while the surroundings of Trp118 are exposed in the kinetic transition state, the surroundings of Trp60 remain native.

G2-052P
Association of cytochrome P450 enzymes is a determining factor in their catalytic activity

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Previous work demonstrated that one cytochrome P450 isozyme can influence the catalytic properties of another P450 isozyme when combined in a reconstituted system. Moreover, many experimental data indicate that P450 interaction is required for catalytic activity even when one isoenzyme is present. The goal of the current study was to examine the possible mechanism of these interactions in more detail. Analyzing recently published X-ray data of microsomal P450 enzymes and protein docking studies, four types of dimer formations of P450 enzymes were found. In case of two dimer types the aggregating partner was shown to contribute to NADPH cytochrome P450 (CPR) binding-a flavoprotein whose interaction with P450 is required for expressing P450 functional activity- of the neighboring P450 moiety. Thus, it was shown that dimerization of P450 enzymes might result in an altered affinity towards the CPR. Two dimer types were shown to exist only in the presence of a substrate, while the other two types exist also without a substrate present. The molecular basis was established for the fact that the presence of a substrate and other P450 enzymes simultaneously determine the catalytic activity. Furthermore, a kinetic model was improved describing the catalytical activity of P450 enzymes as a function of CPR concentration based on equilibrium between different supramolecular organizations of P450 enzymes. This model was successfully applied in order to explain our experimental data and that of other investigators.

G2-053P
Cleavage of the ovine Prion Protein in transfected cell lines

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In prion diseases such as Creutzfeldt-Jakob's disease in man and scrapie in sheep, a key event is conversion of the cellular prion protein (PrPC) into a partly proteinase resistant conformer rich in â sheets (PrPSc). Transgenic mice devoid of PrPC are apparently normal and resistant to prion infection. The physiological role of the prion protein is unknown. In normal brain, as well as in cell-culture, cleavage of PrPC results in separation of the unstructured N-terminal tail and the globular C-terminal two-thirds of the molecule. The cellular site of this cleavage and its possible functional role is unknown. To facilitate studies of the cleavage, different PrP-constructs with green and red fluorescent protein have been used. These, in combination with a panel of monoclonal antibodies, made it possible to trace the full-size molecule and its cleavage products in the neuroblastoma cell lines N2a and SH-SY5Y, and in polarized epithelial MDCK-cells. The present studies show that the full-size fluorescent prion protein was correctly targeted to the plasma membrane, attached by a GPI anchor, and that the cleavage was identical in all three cell-lines. The full-size protein with different glycoforms could be isolated in detergent insoluble pellets, as described for untagged PrPC. Site-directed mutagenesis close to the putative cleavage site at amino acid 113 did not influence the cleavage. Studies of a transfected furin-deficient cell line indicated that cleavage of the prion protein was independent of furin or furin-activated Zn-metalloproteases. Bafilomycin, an inhibitor of proton pumps, seemed to reduce the cleavage, thus indicating that acidic organelles are involved in cleavage of the protein.

G2-054P
Photoregulation of lysozyme activity by photochromism of azobenzene

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Photochromic compounds change their chemical and physical properties reversibly with their structural change by photoirradiation. Therefore the chemical modification of biomaterials by photochromic compounds is useful for effective regulation of their physiological functions. In this study, hen egg white lysozyme (EC3.2.1.17) was chemical modified at specific position by (1) azobenzene-4-carboxylic acid, (2) azobenzene-3-carboxylic acid, and (3) 4-aminoazobenzene. The influence of photochromism of azobenzene moiety on both enzyme activity and photochemical properties was studied. MALDI TOF Mass study revealed that azobenzene-carboxylic acid binds to Lys33, and 4-aminoazobene binds to both Asp 101 and terminal carboxyl group through amide bond. The modified lysozymes exhibit reversible photochromic properties. Upon irradiation of UV light, the trans form turns to cis form and upon irradiation of visible light, the cis form turns back to the trans form. The photochromism of azobenzene leads to the difference in the enzyme kinetics (Michaelis–Menten constant, and maximum initial rate) and fluorescence intensity. NMR study suggests that the photo regulation of the enzyme activity is induced by the minor conformational change in the vicinity of the active site of lysozyme associated with photochromism.

G2-055P
Effect of anti-aggregating agents on the fibrillation process of the amyloid-forming apomyoglobin mutant W7FW14F

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A significant number of fatal diseases are classified as protein deposition disorders, in which a normally soluble protein is deposited in an insoluble form. Although the details of the relationship between protein deposition and disease are not yet completely understood, it is supposed that the aggregation process plays an important role in impairing cellular function. The identification of molecules that inhibit protein deposition or reverse fibril formation may be a critical step toward a better understanding of the physiopathology of amyloid-related human diseases. Preliminary results indicated that some drugs inhibit amyloid fibril formation *in vitro* but the mechanism through which they act is still unclear. In this communication, we examine the effect induced by the presence of tetracycline, quinacrine and chlorpromazine on the fibrillation process and cytotoxicity of the amyloid forming apomyoglobin mutant W7FW14F. Like other amyloid-forming proteins, insoluble mature fibrils formed by the apomyoglobin mutant W7FW14F are not cytotoxic whereas the toxicity of early pre-fibrillar aggregatesis is very high. The effect induced by the tested drugs on the fibrillation process have been examined by atomic force microscopy, light scattering and thioflavin T fluorescence. The cytotoxicity of the amyloid

aggregates was estimated by measuring cell viability. The results show that the examined drugs act as anti-aggregating agents which inhibit the fibril elongation process but not the early aggregation steps leading to the formation of granular aggregates. Thus, this inhibition keeps the W7FW14F mutant in a granular, highly cytotoxic state.

G2-056P
Active site architecture of *E. coli* 1-deoxy-d-xylulose 5-phosphate synthase

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1-deoxy-D-xylulose 5-phosphate synthase (DXS) (EC 2.2.1.7) catalyzes the formation of 1-deoxy-D-xylulose 5-phosphate and CO_2 from pyruvate and D-glyceraldehyde 3-phosphate. This reaction is a common step to three biosynthetic pathways: the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis and the thiamin (vitamin B1) and piridoxol (vitamin B6) biosynthetic routes. DXS has been identified in most bacteria, in plants and in the malaria parasite, *Plasmodium falciparum* but is absent in other eukaryotes, including humans. DXS has been shown to be essential for the bacteria and to catalyze a limiting step (at least in the MEP pathway). For all these reasons DXS has been proposed as an attractive target to develop new antibiotics and antimalarials. To proceed to the rational design of inhibitors structural information on the enzyme is needed as well as identification of the amino acid residues directly involved in catalysis. In this communication the role of conserved residues of *E. coli* DXS was examined by site-directed mutagenesis and kinetic analysis of the purified recombinant enzyme mutants. Significance of these mutations was analyzed by molecular modeling of the catalytic center of DXS based on the structure of other thiamin diphosphate-dependent enzymes.

G2-057P
Structural and functional studies of lipocalin-type prostaglandin d synthase complexed with lipophilic small ligands by X-ray small angle scattering

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Lipocalin-type prostaglandin D synthase (L-PGDS) is a member of the lipocalin superfamily. The tertiary structure of L-PGDS showed an eight-stranded b-barrel architecture, which is similar to that of the other members of lipocalin superfamily such as b-lactoglobulin and retinol-binding protein. The inside structure of L-PGDS is hydrophobic circumstance and is capable of accommodating a large variety of lipophilic low-molecular ligands, e.g., retinoids, bile pigments and thyroid hormones. Such a characteristic of L-PGDS arouses our interest in studying the complex structure of L-PGDS with lipophilic ligands. In the present study, we measured the conformational changes of L-PGDS by the binding of lipophilic ligands with the X-ray small angle scattering (SAXS), and the binding affinities of the lipophilic ligands. SAXS measurements were performed at BL40B2, SAXS station, in SPring-8. The complexes of L-PGDS with retinoic acid (RA), bilirubin (BR) and biliverdin (BV) systems were prepared. We could observe very fine scattering curves for every L-PGDS complexes. Only in the small-angle region, deviation was observed on scattering curves. These results reveal that, when ligands bind to L-PGDS, just a small change occurs on the whole shape of L-PGDS, and the size of the complex molecule is differed by each ligand. Those structural changes may be ascribed for the conformational flexibility of L-PGDS molecule; leading its unique property that L-PGDS exhibits a broad selectivity of ligand binding.

G2-058P
Interaction between low molecular mass polylysine dendrimers and human serum albumin

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Low mass, polylysine dendrimers are rather new branched molecules of antibacterial and antifungal activities. Because of the dendrimers chemical characteristics and suitability for medical applications their biological properties are of great interest. The aim of our work was to investigate polylysine dendrimers impact on protein structure, using HSA as a model carrier protein. For that purpose we used fluorescence technique to observe changes in the protein structure. Our spectrofluorimetry studies showed that dendrimers quench intramolecular fluorescence of the protein as well as influenced the position of the maximum of the spectra. We also observed that changes in pH have impact on dendrimer–protein interactions. Taking everything into account it can be assumed that dendrimers interact with human serum albumin and the strength of that interaction depends on steric distribution of hydrophobic groups as well as on dendrimers composition.

G2-059P
Recombinant expression, purification and NMR analysis of the RING finger domain from the human RBBP6 protein

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RBBP6 is a 230 kDa human protein that has been shown to bind both p53 and Rb *in vivo* [1]. We have recently shown that the protein contains a conserved N-terminal ubiquitin-like domain (unpublished data), which is potentially significant in view of the fact that RBBP6 also contains a conserved RING finger, a domain commonly found in proteins involved in ubiquitination pathway. The RING finger from RBBP6 is atypical in that it contains a C4C4 motif rather than the more common C3HC4 motif. The structures of only two other C4C4 RING proteins have been determined so far, both using heteronuclear NMR [2]. We report here on the recombinant expression and purification of the RING finger from human RBBP6, and the backbone assignment using heteronuclear NMR. An 11.6 kDa fragment containing the RING finger was expressed in bacteria as a GST-fusion, and found to be soluble. Bacteria were grown in minimal media supplemented with 15N-ammonium chloride and 13C-glucose to ensure that the expressed protein was double-labelled for heteronuclear NMR analysis. The protein was purified using a combination of glutathione agarose chromatography, anion

exchange and gel filtration. Mass spectrometry confirmed that the expressed protein incorporated two-bound zinc ions, as is expected, which led us to believe that the protein was natively folded. A complete set of heteronuclear NMR data was collected at 700 and 900 MHz, from which full assignment of the backbone resonances has been achieve. These assignments are now being used as the starting point for determination of the structure of the domain.

Reference

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G2-060P
Attempts for proper folding of recombinant Goalpha protein after inclusion body purification

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The alpha subunit Go protein which belongs to Gi/o family of heterotrimeric G-proteins was purified using GST-tagged pGEX-4T2 system. Large amounts of recombinant protein were produced in the form of inclusion bodies. These inclusion bodies were purified for use in refolding experiments. We tried different protocols that would prevent precipitation of Goα at the refolding step, including step-wise dialysis, use of mild solubilizing agent NDSB (non-detergent sulfobetaine) or reduced/oxidized glutathione mixture. Insoluble Goα was denatured in 8 M urea or 6 M GnHCl and the denaturant was removed by step-wise dialysis. When GnHCl was used as the denaturant, Goα precipitated and came out of solution upon dialysis into TBS. When urea was used, the protein did not aggregate upon dialysis; however, SAXS analysis indicated a misfolded structure for Goα. The refolding protocols using NDSB and reduced/oxidized glutathione also yielded soluble Goα but the protein degraded during dialysis of highly diluted material in large volumes. Attempts to optimize purification of over-expressed recombinant Goα are in progress.

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G2-061P
Comparative evaluation of the artificial chaperone-assisted renaturation of bovine carbonic anhydrase under the effect of various ionic detergents

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Artificial chaperone-assisted refolding has been shown to be an effective approach for improving the refolding yield of some of the denatured proteins. Since identical concentrations of various detergents do not induce similar changes in the protein structures, we arranged to evaluate the artificial chaperoning capabilities of several ionic detergents as a function of charge, structure; and the hydrophobic tail length of the detergent. Although, a minimum structural arrangement between CA and detergents, in the capture step, is required for obtaining reactivation and this minimum structural feature is apparently achieved at different concentrations of various detergents, our results indicate that Carbonic Anhydrase (CA) can be refolded from its denatured state via artificial chaperone strategy using both anionic and cationic detergents. However, the extent of refolding assistance (kinetic and refolding yield) were different due to protein and detergent net charges, detergent concentrations and the length of hydrophobic portion of each detergent. On the other hand, comparison of the far-UV CD and intrinsic fluorescence data with the corresponding reactivation profiles indicate that the various secondary and tertiary structures of the captured protein are established by the detergent type and concentration; however, these structures had minimum effect on the final reactivation yield of the CA. Therefore, the observed differences in the refolding yield were attributed to physical properties of CA-detergent complexes, which can be monitored using ANS fluorescence technique, and/or to the kinetics of detergent stripping by β-cyclodextrin (β-CD) from the protein-detergent complexes which is apparently dependent on the detergent-β-CD association constants and the nature of the partially stripped complexes. Perhaps, β-CD-detergent associations kinetically control the population of partially stripped intermediates which are prone to self association (aggregation).

G2-062P
Neurotoxin II bound to the nicotinic acetylcholine receptor shows little structural changes: a solid-state NMR approach

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Solid-state magic-angle-spinning (MAS) NMR spectroscopy can provide sufficient information for structure determination of a protein. However, a defined micro-environment for each protein molecule is required to obtain suitable spectra. Membrane proteins in two-dimensional crystals or ligands bound to a receptor adopt highly ordered structures which are amenable for these technique. In order to observe the species of interest both uniformly and specifically labeled samples are needed. In this work, we use solid-state MAS NMR to analyze the structure of neurotoxin II while it is bound to the nicotinic acetylcholin receptor (nAChR): Native membranes from the electric organ of *Torpedo californica* which are enriched in nAChR up to 80% were incubated with heterologeously expressed (13C,-15N)-neurotoxin II. The side-chain signal sets of the individual amino acids of the toxin were identified by 2D 13C-13C proton-driven spin diffusion and dipolar-recoupling experiments. The comparison of the observed peaks with resonances obtained from solution NMR data of the soluble toxin shows that most of the expected resonances are observed but significant differences of chemical shifts occur, too. Further experiments are under way to observe long-range distance correlations which provide the basis for structure calculation. The aim is a picture of the conformation of the toxin in the binding pocket of the receptor.

G2-063P
Adsorption of the *Bacillus thuringiensis* Cry4Ba toxin at lipid membrane–water interface: model studies towards nanodevice implications

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Bacillus thuringiensis Cry delta-endotoxins have been shown to kill susceptible insect larvae by forming a lytic pore in the membrane of target midgut epithelial cells. Previously, the 65-kDa trypsin activated Cry4Ba mosquito-larvicidal protein was shown to be capable of permeabilizing liposome vesicles and forming ionic channels in receptor-free planar lipid bilayers. In addition, aromaticity of Tyr-170 located within the alpha4-alpha5 loop in the pore-forming domain was shown to play a crucial role in Cry4Ba toxicity, likely being involved in stabilization of the toxin-induced pore. Here, we have extended our investigations towards the model interactions of the 65-kDa Cry4Ba toxin with dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanoamine (DOPE) lipid monolayers by coupling Langmuir–Blodgett and imaging techniques. The surface pressure was measured as a function of time, during the adsorption of Cry4Ba at the lipid-water interface. To follow the molecule quantity of the Cry4Ba protein or each specific lipid (DPPC and DOPE) at the interface, we measured the deviation of the ellipsometric angle with time. Furthermore, we compared the ellipsometric angle during toxin adsorption at the air-water and lipid-water interfaces. Experiments are in progress and the implications of the Cry4Ba-lipid interactions for nanodevices are discussed.

G2-064P
Investigation of the folding pathway of Cyt2Aa2 insecticidal protein

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Cytolytic (Cyt) toxin is a class of mosquitocidal toxins produced from *Bacillus thuringiensis*. The toxin exhibits specific activity against midgut cells of mosquito larvae *in vivo*. We have characterized the folding pathway of Cyt2Aa2 toxin obtained from *B. thuringiensis* subsp. *darmstadiensis*. The toxin was expressed in *E. coli* and purified by size exclusion chromatography. Steady state unfolding and refolding of the protein was analyzed by monitoring intrinsic fluorescence spectra of toxin in a series of guanidine hydrochloride. The determined unfolding and refolding curves were found to be identical showing three-state transition of native, intermediate and unfolded states. The calculated thermodynamic free energy of transition between native to intermediate and intermediate to unfolded states were around 5–6 and 11–16 kcal/mol, respectively. Kinetic study of unfolding by rapid mixing between protein and various concentration of denaturant has suggested that the transition between intermediate and unfolded states is the rate-determining step with unfolding rate of 5.83×10^{-10}/s. Further characterization of the intermediate state by intrinsic fluorescence, circular dichroism and ANS binding assay has demonstrated specific characteristics of a molten globule-like structure. This revealed molten globule conformation may play a crucial role in structural folding and toxin function.

G2-065P
Mapping of stereoselective binding sites on proteins by capillary electrophoresis and molecular modeling

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Docking of small ligands on protein surfaces provides several alternative conformations characterized by the lipophylicity, binding energy, etc. The reliability of the docking calculations, however, need empirical confirmations. In this study we made an attempt to link model calculations and capillary electrophoresis results. Docking of chiral and achiral compounds were performed by the Sybyl software. The proper configuration that is in accordance to the real placement of the ligand and receptor was chosen by a systematic comparison of data to real experiments. Capillary zone electrophoresis in the presence of the protein (transferrin), were performed to study the interactions. Several approaches have been applied using biopolymers in capillary electrophoresis separations, but the binding mechanism of the enantiomers have not been studied in details. Iron free human serum transferrin is a suitable chiral selector in capillary electrophoresis used in native form in solution. Separation of optical isomers of tryptophan-methylester, tryptophan-ethylester and tryptophan-butylester and various beta-blockers, as well as sympathomimetics were studied in capillary zone electrophoresis applying a distinct transferrin zone prior to sample injection. Changes in the electrophoretic patterns (i.e., in the migration properties) of the molecules reflected the possible interactions with the protein. Molecular modeling was performed to characterize the interaction areas at the surface of iron-free transferrin. The model-calculations are in excellent agreement with the capillary electrophoresis results, which shows that this technique is suitable for the mapping of the interaction sites on protein surfaces.

G2-066P
Prostatic acid phosphatase is dimeric because it is secretory protein

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Significant fraction of a human prostatic acid phosphatase (hPAP) is secreted in secretory granules designated as prostasomes. Catalytic and antigenic properties of enzyme that is exported from the secretory epithelium in prostasomes resemble the properties of the purified hPAP. Prostasomes may be considered as vehicles to deliver the prostatic secretion proteins in close vicinity to membranes of spermatozoa. The kinetic and equilibrium analysis of the reversible denaturation of the prostatic acid phosphatase shows that the folding intermediate of hPAP is an inactive and sensitive to proteolysis monomer. Dissociation into subunits is accompanied with destabilization of beta structure and exposure of hydrophobic surface. Glycerophospholipids of liposomes act as a trap for monomers and inhibit their association into active dimer. The dimeric, native or reactivated forms

of hPAP are not susceptible to proteolysis. The prostatic acid phosphatase is an example of homodimeric protein that gains catalytic activity and stabilization through interactions between subunits. It is likely that, *in vivo*, the dimerization helps to play the biological role of this protein.

G2-067P
Misfolding of cystathionine beta-synthase mutants

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Misfolding of mutant proteins has been implicated in many human diseases including inborn errors of metabolism, and also in homocystinuria due to cystathionine beta-synthase (CBS) deficiency. The aim of our ongoing study is to examine structural and functional consequences of ~30 patient-derived CBS mutations, which have been carefully selected to mirror alterations in different domains of the enzyme. The mutants were expressed in *E. coli* at 37 and 18 °C using a pKK derived vector, the proportion of tetramers and aggregates was assessed by western blotting using non-denaturing gels, and the functional consequences by measuring the catalytic activity. Up to now, we have expressed and analyzed seven mutants in the catalytic and/or heme binding site, four mutants in the dimer–dimer interface and three mutants in the carboxyterminal regulatory domain of the enzyme. Only one mutant in the catalytic/heme binding site formed tetramers at 37 °C while expression at 18 °C enabled correct folding of all but one mutant; despite being correctly folded at 18 °C four mutants remained completely inactive. In contrast, all five correctly assembled mutants in dimer–dimer interface and in the carboxyterminal regulatory region, which were expressed at 18 °C, were partially to fully active (range 36–158% of wild type activity). These preliminary data support the view that misfolding and aggregation may play a substantial role in pathogenesis of homocystinuria and suggest that mutations outside the catalytic site may have less deleterious effect on the enzymatic activity if correct folding of mutant enzyme is achieved.

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G2-068P
Consequence of misfolded proteins: kuru, mad cow disease and Creutzeldt–Jakob disease

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Prions, an acronym for proteinaceous infectious particles, are normally present in brain tissue as harmless cellular proteins; they are particularly unique in that they lack nucleic acids. Prions are able to fold into two distinct conformations, the normal harmless form designated as PrP, in which the tertiary structure contains predominantly alpha helices, and the other, the abnormal variant, referred to as PrPSc, containing beta pleated sheets. The latter form is an infectious agent that initiates a cascading series of reactions resulting in the conversion of normal PrP molecules into the abnormal PrPSc conformation. The expression "one bad apple spoils the batch" describes the transformation of PrP to PrPSc by PrPSc. The more stable PrPSc form is resistant to proteolysis, organic solvents, and high temperatures. The accumulation of disease-causing PrPSc in the brain results in several fatal dementia-like diseases characterized by the loss of brain matter and a resulting spongy appearance; hence, prion diseases are classified as "transmissible spongioform encephalopathies (TSEs)" and include scrapies in sheep (Sc), kuru, a disease once present in the Fore tribe of New Guinea and maintained by the cannibalistic practice of eating the brains of the deceased, bovine spongioform encephalopathy (BSE or mad cow disease), and Creutzfeldt–Jakob disease (CJD), the human counterpart of BSE. Prions may be the cause of Parkinson's, Alzheimer's, amyotrophic lateral sclerosis (Lou Gehring's disease), and other neurodegenerative diseases. In conclusion, anxiety continues around the world regarding MCD and CJD, compounded by their unusually long incubation period, emphasizing the need for on going research on prions as rogue proteins and as new infectious agents.

G2-069P
Thioredoxin system of Streptomyces coelicolor

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Streptomyces genus has become preeminent for genetic research as reviewed recently [1]. The complete genome sequence of *S. coelicolor* [2] revealed several possible thioredoxin genes, and thus *Streptomyces* seems to have a more complex redox system in comparison with other bacterial species. This work describes the cloning, purification and characterization of thioredoxin (TrxA) and thioredoxin reductase (TrxR) from unusual bacterial strain *Streptomyces coelicolor*. The genes of *S. coelicolor* encoding TrxA and TrxR were amplified by polymerase chain reaction, inserted into a pET expression vector and used to transform *Escherichia coli*. The activity of the purified recombinant proteins was demonstrated. ThioredoxinA efficiently reduced insuline and thioredoxin reductase (NADPH-dependent) catalyzed the reduction of 5,5'-dithiobis (nitrobenzoic acid). Enzymatic reactions of *S. coelicolor* TrxR and coenzyme NADPH were dependent upon the presence of *S. coelicolor* TrxA as the intermediate electron carrier with the optimum pH 7.5 and the optimum temperature 29 °C. The kinetic parameters K_m and V_{max} were determinated for thioredoxin system. Homogenous thioredoxin was used for crystallization trials, too. We have determined the crystal structure of TrxA at 1.5 Å resolution. The crystal exhibits the symmetry of space P21212 with unit cell dimension 43.6, 71.8, 33.2 Å.

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G2-070P
TraA and its N-terminal relaxase domain of the Gram-positive plasmid pIP501 show specific oriT binding and behave as dimers in solution

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The multiple antibiotic resistance plasmid pIP501 has an extremely broad host range for conjugative transfer including many Gram-positive bacteria, such as multicellular *Streptomyces*, and Gram-negative *Escherichia coli* [1]. The DNA processing enzyme,

the DNA relaxase TraA and its N-terminal relaxase domain were cloned, expressed and purified as His-tag fusions and were shown to bind to the *tra* promoter region which partially overlaps with the origin of transfer (*oriT*) [2]. TraA and the shortest enzymatically active aminoterminal domain ($TraAN_{246}$) were subjected to band shift assays with single stranded oligonucleotides covering different parts of $oriT_{pIP501}$. $TraAN_{246}$ showed highest binding affinity for an oligonucleotide encompassing the inverted repeat, the cleavage site and additional seven bases upstream (K_D of 26 nM). Chemical cross-linking and SAXS experiments proved that TraA and $TraAN_{246}$ form dimers in solution. Cross-linked proteins were shown to bind to $oriT_{pIP501}$ oligonucleotides. The unfolding of both protein constructs was monitored by measuring the change in the circular dichroism (CD) signal at 220 nm upon temperature change. The unfolding transition of both proteins occurred at around 42 °C. Upon DNA-binding an enhanced secondary structure content and increased thermal stability was observed suggesting an induced-fit mechanism for the formation of the specific relaxase-oriT complex.

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G2-071P
Inhibitory activities of *Helichrysum* taxa on mammalian type I DNA topoisomerase

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DNA topoisomerases are essential enzymes that regulate the conformational changes in DNA topology by catalysing the concerted breakage and rejoining of DNA strands during normal cellular growth (Topcu, 2001). Over the past few years there has been considerable pharmacological interest in these enzymes because the inhibitors of DNA topoisomerases represent a major class of anticancer drugs. Several *Helichrysum* plants were shown to contain antioxidant, antifungal and antibacterial bioactive compounds, which are represented by 27 taxa belonging to 21 species in Turkish flora, out of which 15 are endemic to Turkey (available at IZEF herbarium; www.izef.ege.edu.tr). We investigated the effects of fine extracts prepared from these plants via *in vitro* supercoil relaxation assays of mammalian DNA topoisomerase I using plasmid substrate, pBR322. Cytotoxic alkaloid, Camptothecin, a known inhibitor of eukaryotic topoisomerase I, was used as reference compound throughout the *in vitro* assays. Four of 34 extracts were shown to inhibit the enzyme activity in a dose dependent manner. Quantitative comparison of the supercoiled and relaxed DNA band intensities on agarose jel suggests that these four *Helichrysum* extracts manifest a significant competitive inhibition on mammalian DNA topoisomerase I as manifested in the change of the affinity of the enzyme to its plasmid substrate. The inhibition of topoisomerase I by *Helichrysum* taxa is a significant result as they can be the sources of potential anticancer compounds.

G2-072P
Direct calorimetric measurement of the thermodynamic parameters of amyloid formation

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A great wealth of information on the morphology and characteristic features of the amyloid structure has been accumulated over the last few years. However, knowledge about the thermodynamics of amyloid formation is limited. By introducing a novel method, we directly measured the heat capacity and enthalpy change of the amyloid formation by isothermal titration calorimetry. Amyloidogenic proteins and peptides such as β_2-microglobulin and polyglutamine were used to extend amyloid fibrils in a seed-controlled reaction in the cell of the calorimeter. From the observed thermodynamic parameters we could infer important structural features of the amyloid fibrils such as the extent of surface burial, the level of internal packing of side-chains, and the possible presence of unfavorable side-chain contributions. In comparison to native globular proteins the results outline the altered weights of the various interactions in the stability of the amyloid structure and a different balance of the enthalpy–entropy contributions.

G2-073P
Comparative study of legume and non-legume lectins in presence of TFE and HFIP

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All legume lectins share a common tertiary structure although their quaternary structures differ considerably. The quaternary organization of these lectins may be of prime importance for their function *in vivo*. Despite the high homology and similarity in their monomeric structures, members of legume lectin family adopt distinct modes of oligomerization. It has been suggested that slight differences in tertiary structure of the monomer may cause appreciable changes in quaternary associations. In our present study we have tried to compare the folding pattern and stability of two similar tetrameric legume lectins, concanavalin (Con A) and soybean agglutinin (SBA). Also the comparison of these with a non-legume lectin, Jacalin, has been made in order to understand how acid-unfolded states of lectins from the same family differ from each other and from a lectin of a different family under the influence of two different fluoroalcohols, trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP). The effect of alcohols on proteins is useful for considering how structures are stabilized in an aqueous environment. Near- and far-UV CD spectroscopy, intrinsic as well as extrinsic fluorescence studies show that Con A seems to be more labile than SBA and that jacalin behaves in a manner similar to that of SBA. The higher conformational stability of SBA and jacalin in comparison with Con A may be attributed to their degrees of subunit interactions.

G2-074P
Computational studies of the interactions of calmodulin with antagonists

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Calmodulin (CaM) is a Ca^{2+} receptor in eukaryotic cells that regulates many important physiological processes. The binding of calcium ions induces a conformational change in CaM, which converts it into an active form that can bind a wide variety of targets. CaM is the object of much interest in pharmaceutical research because it can also bind antagonists. We report on the structure, dynamics and non-bond interactions of CaM bound to two antagonists: trifluoroperazine (TFP), which belongs to the phenothiazine class and DPD an arylakylamine-type antagonist. These antagonists are used for treating brain ischemia and as anti-mitotic drugs (DPD and TFP). We investigated the associative features of these CaM-antagonist complexes using a computational approach that features realistic explicit solvation conditions in the presence of physiological concentrations of counterions. After minimization, heating and equilibration dynamics, we analyzed H-bonding, coulombic, van der Waals and hydrophobic interactions, which were shown to differ in the various complexes.

G2-075P
Effect of temperature on the refolding yield of α-amylase through artificial chaperone-assisted technique

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Nowadays, the development of a protocol for the efficient refolding of the recombinant proteins has become an important issue because a variety of genetically engineered proteins for use in medical and bioindustrial areas are usually produced in inactive aggregate forms called inclusion bodies. Our laboratory has investigated the refolding of GuHCl denatured α-amylase by a new strategy using the artificial-chaperone-assisted two-step mechanism. In the first step, the protein is captured by a detergent and in the second step cyclodextrin strips the detergent away from the protein, allowing proper refolding. Comparison of different kinds of capturing agents (detergents) showed that more recovered activity was observed with ascending the number of methylene groups of the aliphatic tail of the detergent from 12 carbon atoms in the presence of DTAB (refolding yield of 66%) to 14 and 16 carbon atoms for TTAB (refolding yield of 71%) and CTAB (refolding yield of 82%) respectively. This enhancing yield is probably due to the binding constant of detergent and the cyclodextrin. The results also showed that in all cases β-CD works better than α-CD in the stripping step although the values of the complexation constants for α-CD-detergents is much higher than for β-CD in each case. Thus, the decrease in the refolding yields could be attributed also to the rate of detergent stripping from the protein-detergent complex. To optimize the refolding yield further the effect of temperature on refolding yield was also evaluated. Our results indicated that by lowering the temperature of the refolding environment in the time of cyclodextrin addition, the refolding yield reaches its maximum (100%) mainly by suppressing the aggregation of the protein during stripping step. The details will be discussed in the presentation.

G2-076P
Structural dynamics and binding site mapping of β-secretase

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β-secretase (BACE) is a critical enzyme in the production of β-amyloid, a protein that has been implicated as a potential cause of Alzheimer's disease (AD). 3D structural information about β-secretase can facilitate the rational design of small molecule and potent inhibitors. Crystal structures of BACE/inhibitor complexes have also revealed much about the binding sites and the nature of protein–ligand interactions that prompted both academic and industrial research groups to design new peptidomimetic or non-peptidomimetic inhibitors. Since the dynamic properties of a protein have a profound effect upon its functional behavior and their characterization is complementary and synergic to structural studies we performed a thorough analysis into the molecular movements related to ligand binding. Molecular dynamics calculations were performed to characterize the movements of structural domains and protein–ligand interactions. Finally, we concluded that the protonation state of the active site is crucial in ligand binding and can change parallel to protein conformation upon binding.

G2-077P
Mutation of Glu367 affects the quaternary structure of goose delta-crystallin

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Delta-crystallin is a soluble structural protein in avian eye lenses that confers special refractive properties. This protein also belongs to one of the taxon-specific families of enzyme crystallins which sharing high amino acid sequence identity with argininosuccinate lyase. Previous studies have revealed that in the presence of low GdnHCl concentration, tetrameric delta-crystallin undergoes reversible dissociation via dimers to a monomeric molten globule intermediate state. In the present study, the role of Glu 367 in protein assembly and conformational stability has been studied. In the structure, this residue was located in the interface of double dimers, close to the 280' loop of neighbor subunit. This loop has been assumed to be important for catalysis. Replacement of this residue by alanine did not produce any gross structural changes, as judged by circular dichroism analysis, tryptophan fluorescence and ANS spectrum, but about 60% enzyme activity was lost. This mutant protein has about five degree lower in T_m value than wild type, but remaining similar stability to GdnHCl. Interestingly the dimeric form was observed for the mutant, as judged by sedimentation velocity. These results indicate that the interactions offering by Glu 367 in the interface of goose delta-crystallin is important for two dimers assembly and conformational stability.

G2-078P
Oxalate catabolism by *Oxalobacter formigenes*: formyl-CoA transferase is a truly interlocked dimer, and Oxalyl-CoA decarboxylase has the classical Thdp-binding fold with CoA bound in the FAD-site of aceto-hydroxyacid synthase and pyruvate oxidase

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Oxalobacter formigenes is an obligate anaerobe colonizing the human gastrointestinal tract, and employs oxalate breakdown to generate ATP. This process involves two coupled enzymes and a membrane-bound oxalate:formate antiporter. Formyl-CoA transferase first catalyses the transfer of CoA from formate to oxalate, activating the oxalyl moiety for thiamine-dependent decarboxylation by the second enzyme in the pathway, oxalyl-CoA decarboxylase. Formyl-CoA transferase is the first Class III CoA-transferase for which a 3D-structure has been determined [1]. It has a novel fold and a very striking assembly of the homodimer. The enzyme monomers are tightly interacting and are interlocked. This fold requires drastic rearrangement of ~75 residues at the C-terminus for formation of the dimer. Kinetic characterization of formyl-CoA transferase and site-specific mutants suggested that catalysis proceeds via a series of anhydride intermediates. This mechanistic proposal is corroborated by the crystallographic observation of an acyl-enzyme intermediate that is formed when formyl-CoA transferase is incubated with oxalyl-CoA [2]. This dimer structure and catalytic mechanism should be conserved in the Class III CoA-transferase family. The crystal structure of tetrameric oxalyl-CoA decarboxylase in complex with ThDP and CoA has now been solved to 1.7 Å from a merohedral twin-crystal. The monomer shows the three-domain organization and the conserved active site commonly found in ThDP-dependent enzymes. The electron density map shows CoA to be hydrolyzed at two sites; the pantetheine tail as well as the 3-phosphate of the ribose is missing.

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G2-079P
Design of a potential-energy function for protein folding studies

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We present a united-atom force field for protein folding which is obtained by optimizing the parameters of the OPLS potential-energy function with the Generalized-Born/Surface Area (GB/SA) solvation model by the linear-programming method. Optimization is directed to satisfy the following two requirements: (i) the native-like conformation is the lowest in energy and (ii) the energy increases with decreasing similarity to the native structure. 1L2Y and 1BDD are chosen as training proteins. The decoy structures of training proteins are generated by global and local conformational space annealing (CSA) methods. Results of the calculations for these two proteins are presented.

G2-080P
Calcium regulates the stability of the glyconeogenic metabolon in heart and skeletal muscles

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In recent years, we have discovered that muscle fructose 1,6-bisphosphatase (FBPase) – a key regulatory enzyme of glycogen synthesis from non-carbohydrates – is located on the Z-line of the sarcomere. Using confocal microscopy, the real-time interaction analysis and the kinetic experiments we have presented evidence on the existence of a triple aldolase–FBPase–α-actinin complex, in which binding of muscle FBPase to muscle aldolase desensitizes the former enzyme toward its main inhibitor – AMP. We have also shown that interaction of the two enzymes results in the substrate channeling. Taking these into account, we have postulated the existence of glyconeogenic metabolon in the region of the Z-line of vertebrates' striated muscles. Since the association of many enzymes with subcellular structures is regulated by the physiological state of the cells, we have studied the effect of elevated Ca^{2+} concentration on the stability, activity and subcellular localization of aldolase–FBPase complex. Results of the experiment revealed that calcium decreases both the affinity of FBPase to aldolase and α-actinin. This destabilizatory effect of Ca^{2+} results in the release of muscle FBPase from the Z-line and free form of the enzyme is strongly inhibited by calcium ions. On the other hand, the increased concentration of Ca^{2+} causes the accumulation of aldolase within I-band, the region of the postulated glycolytic complex occurrence. These results might suggest the presence of a calcium-dependent mechanism of glyconeogenic metabolon disintegration and of glycogen synthesis inhibition (and, simultaneously, glycolysis acceleration) during muscle contraction.

G2-081P
Expression, purification and structural prediction of the ets transcription factor ERM

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The PEA3 group of the Ets family is made up of PEA3, ER81 and ERM, which are transcription factors of about 500 residues. They are highly conserved in the ETS DNA-binding domain as well as in the two transcriptional activation domains. These factors are involved in many developmental processes and regulate cancer via metastasis, as in the case of some breast tumors. In this study, we describe the overexpression, purification, and characterization of human ERM in *Spodoptera frugiperda* (Sf9) cells, using the baculovirus system. Overexpression of ERM was confirmed by measurement of SDS-PAGE and Western blot analyses. Two-step purification by affinity chromatography led to a highly stable

protein. Electromobility shift assays showed that this purified protein is functional since recognizing specific Ets DNA-binding sites. We then performed a global structural analysis of ERM by circular dichroism and infrared spectrometry methods and compared it with the result of the current structural prediction algorithms. Our study indicated that ERM contains a highly structured ETS-domain and suggested that each of the N- and C-termini transactivating domains also contains an a-helix. The 250 residue central domain however seemed to be very weakly structured.

G2-082P
Surface plasmon resonance; a method for assessment of biological and immunological activity of hepatocyte growth factor

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Hepatocyte growth factor (HGF) has been widely studied for its unique regenerative properties on injured epithelial cell layer. There are variants of HGF, which have defective biological activity and it is therefore important to evaluate the quality of HGF. We intended in the present study to develop a rapid method to investigate the biological as well as immunological activity of HGF. Surface plasmon resonance (SPR) is an optic and label-free method for detection of molecules by ligand–ligand interaction. Using SPR we immobilized the sensing surfaces in the flow cell channels with three ligands; recombinant human HGF receptor chimera, monoclonal anti-human HGF and neutralizing anti-human HGF antibodies. The fourth channel contained the dextran matrix in which the ligands were immobilized. Seven batches of human recombinant HGF and HGF from different body fluids were analysed, showing that some batches of HGF had affinity to all four channels. The effect and mechanism of HGF activity was also studied in a model of injured epithelial cell layer (CCL-53.1). The migration of epithelial cells towards the injured area was significantly increased dose-dependently by the SPR responsive HGF. The cells did not respond to the other heparin-binding growth factors and the migration of cells was inhibited by anti-human HGF antibody. SPR responsive HGF accelerated also the hair growth in mice after subcutaneous injection dose-dependently. The non-responsive HGF (showing no affinity to SPR flow cell channels) was not biologically active, but was immunoreactive in ELISA. We conclude that the SPR method used in this study might distinguish the immunologically as well as biologically active HGF from the other variants accurately.

G2-083P
The discrimination of tyrosine residues in ribonuclease A by picosecond time-resolved fluorescence spectroscopy

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Ribonuclease A (RNase A) is a well-known model protein for protein stability studies. However, its low fluorescence quantum yield and small variations during unfolding has hindered the use of fluorescence for wild-type RNase A. The fluorescence intensity of RNase A (I(t)) is the sum of fluorescence intensities (Ii(t)) of six tyrosine (tyr) residues. Ii(t) is proportional to the concentration of excited-state tyr ([Ai*]0) at $t = 0$ and respective fluorescence decay time (taui). Fluorescence decay of RNase A at 25 °C (pH 7) is triple-exponential (tau1 = 1.7 ns, tau2 = 180 ps and tau3 = 30 ps). On hexa-normalization of its pre-exponential coefficients (ai), a distribution of 1:1:4 was observed, indicating that four tyr are strongly quenched, one is partially and one is slightly quenched. Further measurements from 20 to 80 °C showed that the decays remain triple-exponential at 80 °C. tau1 decreases to 0.83 ns at 80 °C whereas tau2 and tau3 increase to 330 and 80 ps, respectively. ai changes from 1.1:4 at 25 °C to 1:3:2 at 80 °C, indicating two strongly quenched tyr at 25 °C are partially quenched at 80 °C. The quenching mechanism in RNase A was rationalized on the basis of a photo-induced electron transfer (kET) from phenyl to disulphide bridges (-SS-), where kET is exponentially proportional to the distance (R) between phenyl and -SS-. R values were calculated for native and unfolded RNase A and the following assignment was proposed. Tyr-76 was assigned to tau1, Tyr-73 to tau2, Tyr-115 to tau3, Tyr-25 to tau3 at 25 °C and tau2 at 80 °C, and either Tyr-92 or Tyr-97 to tau2 at 80 °C. These results open the door to tracking conformational changes occurring simultaneously in different parts of the protein during protein unfolding.

G2-084P
Biochemical characterization of chimerical mutant forms of human phenylalanine hydroxylase: a contribution to the understanding of enzyme stabilization

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Most forms of phenylketonuria (PKU; OMIM 261600) and hyperphenylalaninemia are caused by mutations in the phenylalanine hydroxylase gene (*PAH*). The human enzyme (hPAH; EC 1.14.16.1) has proven to be very susceptible to mutations all over its structure leading to a decrease in its enzymatic activity and/or stability. Till now only a very limited number of chimerical mutant forms of hPAH showed an increase in its specific enzymatic activity. Using site-directed mutagenesis (Quickchange II) of wild-type hPAH cDNA, chimerical hPAH proteins were design in order to alter: (1) the protein surface charge (D145K, D151K, E181K and E360K), and (2) the tendency of free sulfhydryl groups to undergo oxidation during long-term storage (C29S and C29D). The recombinant enzymes were produced in a prokaryotic expression system (pTrc-His) as a fusion protein possessing a 6xHis N-terminal peptide, which allowed its purification using immobilized metal affinity chromatography (IMAC). The purified proteins were characterized regarding its specific activity and stability. To monitor conformational changes related with enzyme activity modulation, the purified proteins were subjected to size exclusion chromatography and thermal stability assays. The mutant C → D proteins did not show any significant differences when compared to the wild-type protein. However, the mutant D → K hPAH proteins proved to be more susceptible to enzyme activity modulation mainly through an effect over its oligomeric profile.
The obtained results showed that using protein engineering it is possible to modulate human enzyme stability, which could be used in enzyme therapy. Moreover, the characterization of the

above chimerical hPAH proteins will contribute to the understanding of the wild-type conformational stability.
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G2-085P
Protein–protein and RNA–protein interaction in the assembly *in vitro* complexes from potato virus X RNA, coat and movement proteins

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Potato virus X (PVX), a type member of the potexvirus genus, is a filamentous positive RNA plant virus. It has been proposed that genome of PVX moves from cell to cell as assembled virions or as a non-virion complex (vRNP) consisting of the viral RNA, CP and TGB1, one of three movement proteins encoded in so called triple gene block (TGB). In the present work we examined the complexes assembled *in vitro* from PVX RNA, TGBp1 and CP. The structure and *in vitro* translatability of complexes assembled at different molar ratios of PVX RNA:CP and RNA:TGBp1 were characterized. Recently we have shown that virion PVX RNA was non-translatable *in vitro*, though PVX RNA could be converted into translatable form after interaction with TGBp1 or after PVX CP *in situ* phosphorilation (Atabekov et al., 2000, 2001). Here, it was found that interaction of TGBp1 with disaggregated PVX CP resulted in insoluble TGBp1-CP complexes production. In the presence of PVX RNA soluble single-tailed particles (TGBp1-RNA-CP or RNA-CP) were assembled. It was suggested that RNA-mediated co-assembly of soluble triple TGBp1-RNA-CP complexes could proceed in two-steps. At the first step, assembly of non-translatable single-tailed helical RNA-CP complexes occurs containing the 5'-end of PVX RNA encapsidated within the helical ''head''. Next is the binding of TGBp1 to CP subunits exposed at the end of the polar ''head'' or by PVX CP *in situ* phosphorilation included in vRNP, resulting in conversion of single-tailed particles from a non-translatable into a translatable form.

G2-086P
BRCA1 missense mutations destabilize its structure and alter the binding of the helicase BACH1

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Loss-of-function germ-line mutations in the 1863-residue BRCA1 gene product predispose carriers to breast/ovarian cancer. The classification of more than one hundred missense mutations located in BRCA1 BRCT-domain, deposited at Breast Cancer Information Core (BIC), remains an important issue in Cancer Genetics. Although functional studies have shown that several of them lead to loss of function *in vitro*, their role in oncogenesis has to be established. Very few data exist addressing the effects of these mutations upon the structure and the stability of the protein. We have shown that thermal denaturation of the BRCT region involves a partly unfolded intermediate that is structurally very similar to the native. Extending our studies to explore stability of the mutants, we have constructed and expressed G1738E, G1738R, G1788V, M1783T, V1833M, M1775R and R1699W BRCT mutants. Some of them have already been linked to cancer predisposition, while the rest need to be more extensively studied in order to be classified as deleterious or not. Only the mutants V1833M, M1775R and R1699W were obtained in large amounts and in a soluble form. Mutation M1775R does not alter the secondary structure of the BRCT domain and denatures to an intermediate state that also has similar secondary structure as the denatured wild type protein. Yet, the thermostability of the mutant protein is 11 °C lower, indicative of the reducing of thermodynamic stability of the mutant with respect to the intermediate. The thermodynamic characterization of the remaining mutants is in progress using a combination of biophysical techniques such as differential scanning calorimetry, circular dichroism and isothermal titration. Recently, it has been demonstrated that the BRCT domain interacts with the phosphorylated BRCA1-associated carboxylterminal helicase protein (BACH1). The region that is responsible for this interaction corresponds to aminoacids 985–1001 of the BACH1 protein that include the phosphorylated Ser990. The binding of the BACH1 phosphopeptide results to a more thermostable protein. The titration data were fitted using nonlinear least squares analysis, leading to K_d = 1.4 μM, ΔH_0 = –19.7 kcal/mol and a stoichiometry (N) = 1.12.

G2-087P
Production of thermostable protease from *Bacillus licheniformis* LBBL-II isolated from traditionally fermented locust beans (*Parkia biglobosa*) in south-western Nigeria

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Thirty-five bacillus strains were isolated from traditionally fermented locust beans (*Parkia biglobosa*) in Akure, SW Nigeria. Twelve of the *Bacillus* strains were identified as *Bacillus licheniformis*. All the *Bacillus* strains were tested for proteolytic activity on skim milk agar.*B. licheniformis* strain LBBL-II exhibited the highest proteolytic activity with an average area of clear zone measuring 960 mm^2. The optimum pH and temperature of protease from *B. licheniformis* LBBL-II were 8.0 and 60 °C, with casein as substrate. The enzyme was stable between pH 7.0 and pH 10.0. This protease was almost 100% stable at 60 °C after 60 min of incubation.*B. licheniformis* LBBL-II seems to be a promising alternative microorganism that can be used as starter culture in the production of fermented locust beans.

G2-088P
Comparison of pyruvate kinase variants from human breast tumor and normal breast

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Pyruvate kinase isozymes in human breast tumor tissue were compared in this study with normal human breast tissue. Two

forms of pyruvate kinase present normal and tumor human breast were purified by ammonium sulphate precipitation, dialysis, gel filtration, ion exchange and affinity chromatography. Molecular weight of the native enzyme was determined. Presence of pyruvate kinase activity was examined in the normal and tumor breast tissues. Pyruvate kinase was purified with Sephadex DEAE-50, Sepharyl S-200 and Blue Sepharose CL-6B chromatography. Spectrophotometric methods were used to determine activities of pyruvate kinase. Molecular weight of fraction I and II as determined by gel filtration on Sepharyl S-200 were 135 000 Da, 260 000 Da in normal breast tissue and 72 000 Da, 250 000 Da in tumor breast tissue, respectively. Fraction I and II of pyruvate kinase may be purified approximately 1,591-fold, 636.4-fold in normal breast tissue and 219-fold, 318-fold in tumor breast tissue, respectively. Pyruvate kinase activity in tumor tissue was found higher than normal tissue. Only tumor fraction II showed a tumor-specific sensitivity to L-cysteine. L-phenylalanine inhibited both fraction I and II of normal breast and fraction I of tumor breast, but not fraction II of pyruvate from tumor. ATP inhibited normal and tumor fraction I of pyruvate kinase. The influence of ATP on the enzyme activity from normal and tumor fraction II depended on its concentration. It was thought that isozymes of pyruvate kinase from human breast tissue might be M1 and M2 isozymes when compared with those of other tissue pyruvate kinase isoenzymes. Fraction II from breast tumor represented different sensitivity to L-cysteine, L-phenylalanine and specific activity in comparison with fraction II from normal breast. Different kinetic behaviour of fraction in the human breast tumors may support the concept of an isozyme shift.

G2-089P
The molecular basis for the green to red conversion of the fluorescent protein from *Dendronephthya* sp.

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The green fluorescent protein (GFP) from jellyfish *Aequorea victoria* and its homologs from corals have a broad application in biotechnology as visual reporters for events in living cells. Their applicability in cellular biology is due to autocatalitic synthesis of the chromophore from amino acids inside the protein shell. Thus, these reporters do not require any external agents for the fluorescence appearance. In general the chromophore structure defines spectral properties of the protein whereas alterations in the cromophore environment provides the shift of absorption/emission maxima of no more than some tens of nanometers. In this work we determined the molecular basis of green (emission maximum at 504 nm) to red (emission maximum at 575 nm) conversion of the fluorescent protein from the coral *Dendronephthya* sp. (DendFP) under UV-irradiation. UV-illumination causes fragmentation of the polypeptide backbone of DendFP, as seen in SDS-PAGE gels. To clarify the chemical nature of this phenomenon DendFP was subjected to proteolytic digestion with trypsin. The chromophore-containing peptide was isolated from the tryptic digest by HPLC. The structure of the chromopeptide containing the ''red'' chomophore was determined by ESI, ESI/MS/MS mass-spectrometry and NMR. The data obtained suggest that the photoinduced green-to-red conversion results in the cleavage of Dend FP polypeptide chain between Leu 64 and His 65 and double bond formation in the side chain of His 65. Consequently, the red shift is explained by the extended p-electron system, which involves the newly formed C=C double bond and imidazole group of His 65. Recently, the analogous photoconversion was found in a fluorescent protein from *Trachyphyllia geoffroyi* (Kaede). Therefore, DendFP can be attributed to the Kaede subfamily of GFP-like proteins in the classification based on the chromophore structure.

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G2-090P
Calorimetric and spectroscopic study of the conformational stability of Shiga toxin B-subunit

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The conformational stability of Shiga toxin B-subunit (STxB), a bacterial homopentameric protein from *Shigella dysenteriae* involved in cell-surface binding and intracellular transport of the toxic A-subunit, has been characterized by differential scanning calorimetry, circular dichroism and infrared spectroscopy under different solvent conditions. It is shown that the thermal folding/unfolding of STxB is a protein concentration and pH-dependent process involving a highly reversible cooperative transition between folded pentamer and unfolded monomers in aqueous conditions. Structural thermodynamic calculations show that the stabilization of the STxB pentamer is primarily due to the interactions established between monomers rather than intramonomer interactions. Trifluoroethanol-induced conformational changes of STxB give rise to a rich-helical reversible intermediate state less compact than the native form and with no significant tertiary structure. The highly cooperative trifluoroethanol-induced transition from the native pentamer conformation to a monomeric conformation is centered at 25% (v/v) trifluoroethanol concentration. Thermal stability of STxB was investigated at various protein and trifluoroethanol concentrations and was successfully fitted to the dissociation/unfolding two state model. At higher trifluoroethanol concentrations, no cooperative transitions were detectable. Exposure to the solvent of buried apolar surface area in presence of trifluoroethanol could explain the appearance of this monomeric structured conformation.

G2-091P
What is the cause of anomalous phenomena in actin solutions with low guanidine hydrochloride concentration?

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One of the basic methods for study protein folding is the investigation of proteins unfolding – refolding induced by different denaturing agents. Guanidine hydrochloride (GdnHCl) is commonly used for these purposes. Nonetheless the exact mechanism of GdnHCl interaction with proteins is not known. In the course of examination of actin unfolding induced by GdnHCl we have found anomalous phenomena in actin solutions with low GdnHCl concentration such as the increase of parameter $A = I_{320}/I_{365}$, which characterizes the fluorescence spectrum position, the increase of intensity of light scattering and the increase of anilino-

naphthalene sulfonate (ANS) fluorescence intensity. We explain all these phenomena by actin macromolecules aggregation in the presence of low GdnHCl concentration. It is due to the interactions of NH2 group of GdnHCl cation (GuH+) with C=O group of glutamic and aspartic acids, or glutamine and asparagine, located on the surface of actin macromolecule. The increase of GuH+ ions connected with actin leads to the increase of positive charged groups on the protein surface. At definite concentration of GdnHCl initially negative charged actin macromolecule as a whole, becomes neutral. Under such conditions the protein macromolecules associate. This leads to the increase of light scattering and ANS binding with these associates. The further increase of GdnHCl concentration leads to the appearance of positive charge on the protein surface that prevents association.

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G2-092P
Human ribosomal proteins S13 and S16: expression, purification and refolding

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Recombinant eukaryotic ribosomal proteins are valuable tools to investigate their interactions with various RNAs (ribosomal, messenger, etc.). We expressed, purified and refolded recombinant human ribosomal proteins S13 and S16 that are homologues of the prokaryotic ribosomal proteins S15 and S9, respectively. Total cDNA obtained by reverse transcription of human placental poly(A)+ -RNA with an oligo(dT)12-18 primer was amplified by PCR using pairs of specific primers for the mRNAs of the proteins S13 and S16. PCR products were inserted into pET15b vector (Novagen) on *Nde*I and *Bam*HI sites. The resulting pET-S13 and pET-S16 plasmids coding for ribosomal proteins S13 and S16 fused with N-terminal (His)6-tag were transformed into *E. coli* BL21(DE3) cells. Proteins were accumulated in inclusion bodies mainly. After isolation, proteins were dissolved in denaturant, affinity purified on a Ni-NTA agarose column and dialyzed against buffer solution (pH 7.5) to refold proteins in native state. Purity and integrity of the proteins were proved by SDS-PAGE. Folded state of the proteins was evidenced from circular dichroism (CD) spectra. The content of the secondary structure elements of the proteins was calculated from the CD spectra using program SELCON3. The α-helices/β-sheets contents were determined as 43 ± 5/11 ± 3% and 22 ± 5/24 ± 5% for the proteins S13 and S16, respectively. These data well correlate with the contents of the α-helices and β-sheets in the secondary structures of their prokaryotic homologues. By measuring CD of the recombinant proteins at 222 nm under various pH and concentrations of urea, optimal conditions for stable native states of the proteins were found.

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G2-093P
Interaction of human GTP cyclohydrolase I with splice variants

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Tetrahydrobiopterin is an essential cofactor for aromatic amino acid hydroxylases, ether lipid oxidases and nitric oxide synthases [1]. Its biosynthesis in mammals is regulated by the activity of the homodecameric enzyme GTP cyclohydrolase I (GCH). We co-expressed inactive C-terminal splice variants of human GCH [2] together with the wild type enzyme in mammalian cells, in order to characterize their biological role. Real-time PCR confirmed that GCH mRNAs expressed in bicistronic vectors at similar levels around 1 per 100 million 18 S ribosomal RNA. Western blots of protein extracts revealed that GCH levels were reduced in the presence of splice variants. Splice variants were also expressed as N-terminally S-tagged fusion proteins to allow specific detection. Commensurate with these findings, the GCH activity obtained for wild type enzyme was reduced 2.5-fold through co-expression with splice variants. Interestingly, Western blots of native gels suggest that C-terminal variants are incorporated into decamers. Purification of bacterially expressed splice variants will allow us to study their interaction with wild type GCH and their effect on enzyme stability and activity in more detail.

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G2-094P
Thermal stability and enzymatic activity of subunit hybrids of tetrameric alcohol dehydrogenases from the extreme thermophile *Thermoanaerobacter brockii* and the mesophile *Clostridium beijerinckii*

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To examine the role of inter-subunit interactions in maintaining enzymatic activity and conferring thermal stability to oligomeric enzymes, we prepared subunit hybrid enzymes of highly homologous secondary alcohol dehydrogenases (ADHs) from the extreme thermophile *Thermoanaerobacter brockii* (TbADH) and the mesophile *Clostridium beijerinckii* (CbADH). Although the enzymes share 75% amino acid sequence identity, they differ greatly in their thermal stability. TbADH has the respective $T_{½}^{60\text{min}}$ (temperature at which 50% of the enzymatic activity is lost after 60 min) and T_m (thermal denaturation midpoint monitored by the change in CD) values of ca. 93 and 98 °C, whereas the respective values of CbADH are ca. 63 and 70 °C. A detailed comparison of the high-resolution X-ray structures of the two recombinant enzymes (at resolutions of 2.5 and 2.05 Å, respectively) suggests that the enhanced thermal stability of TbADH is due to the strategic placement of structural determinants that strengthen the interface between its subunits. To obtain *in vivo* hybrids of the two ADHs in reasonable quantities, we constructed a hybrid expression vector that contains, in tandem, the coding regions of both CbADH and tagged-TbADH and used it to transform *E. coli* cells. The recombinant ADH monomeric subunits overproduced in *E. coli* were assembled *in vivo* into tetramers that could be separated by chromatographic procedures. The process yielded a mixture of the entire repertoire of tetrameric ADH hybrids – namely $[TbADH]_4$:$[CbADH]_0$, $[TbADH]_3$:$[CbADH]_1$, $[TbADH]_2$:$[CbADH]_2$, $[TbADH]_1$:$[CbADH]_3$, $[TbADH]_0$:$[CbADH]_4$. The homotetramers, $[TbADH]_4$:$[CbADH]_0$ and $[TbADH]_0$:$[CbADH]_4$, thus obtained were indistinguishable from the respective native TbADH and CbADH in their enzymatic activity and thermal stability. Conversely, each ADH heterotetramer showed distinct enzymatic properties and altered thermal stability that could be attributed to its unique composition and structure. A comparison of the properties of the hybrid ADHs with those of the two wild-type enzymes indicated that the extreme thermal stability of TbADH is conferred largely by the inter-subunit interactions.

G2-095P
Structure of an s-layer protein SbsC

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Crystalline bacterial surface layers (S-layers) represent the outermost cell-envelope component of many prokaryotes. They are composed of identical protein or glycoprotein subunits with the ability to self-assemble into two-dimensional crystalline arrays exhibiting either oblique, square or hexagonal lattice symmetry with a centre-to-centre spacing of the morphological units of 3.5–35 nm. Due to their high degree of structural regularity, S-layers represent interesting model systems for studies on structure, genetics, functions and dynamic aspects of assembly of supramolecular structures. The SbsC protein from *Geobacillus stearothermophilus* consists of 1099 amino acids including a 30-amino-acid leader peptide. For obtaining 3D-crystals and determining the structure–function relationship of distinct segments of SbsC, N- and C-terminal deletion mutants were produced. Crystals of a C-terminal deletion mutant, $rSbsC_{31–844}$, were obtained. They crystallized in space group $P2_1$ and diffracted to ca. 3 Å at our home source and at the synchrotron. Knowing unit cell dimensions, the Matthews coefficient was calculated to be 3.5 Å/Da (one molecule in asymmetric unit) with an estimated solvent content of 63%. Soaking with various heavy atom salts gave good platinum, lead and osmium derivatives. Only MIRAS with all available derivatives led to the interpretable electron map where secondary elements were recognized. Determination of the structure is in progress. The secondary structure prediction revealed that $rSbsC_{31–844}$ consists of 35% β-sheets and 23% α-helices, which was also confirmed with circular dichroism measurements.

G2-096P
Investigation of d-amino acids oxidase of *Aspergillus niger* R-3

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The investigation of D-amino acid oxidases is very actual in biotechnological respect, as they can be used for receiving the L-isomers of amino acids from racemic mixtures by destroying the D-isomers, for controlling the degree of purity of natural amino acids preparations, for the production of different ketoacids from D-amino acids that can be used in the diagnosis of renal insufficiency. High D-amino acid oxidase activity is revealed in mold fungi, producer of citric acid, *Aspergillus niger* R-3, when the culture was grown on the waste product of the sugar industry – molasses. Optimal conditions of growth in synthetic media have been developed, in which the greatest activity of enzyme will display. It has been proved, that the culturing the fungi in the media with reduction concentrations of glucose and methionine racemat produces the greatest enzyme activity. Method of purification of D-amino acid oxidase has been developed, which includes the treatment by $(NH_4)_2SO_4$, gel filtration on sephadex G-200, chromatography on sephadex A-25 and hydroxy-apatite. As a result two ezoenzymes of D-amino acid oxidase were discovered, each with molecular weight 187 000 Da. This method permits to obtain izoenzymes, with the degree of purification 155 and 115, and the yield 29% and 22% accordingly. Each isoenzyme consists of four subunits with molecular weight 46 700 Da. The isoenzymes are carbothiolic and contrast to animal enzyme, the D-amino acid oxidase from *Aspergillus niger* R-3 requires no exogenous cofactors such as FAD and Fe^{2+} ions. Thus the results of these researches showed, that the mold fungi *Aspergillus niger* R-3, producer of citric acid, can be used also as an effective producer of D-amino acid oxidase.

G2-097P
Comparative molecular dynamics simulations of mesophilic and cold-adapted homologous enzymes

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In recent years, there has been increased interest in the origin of protein cold adaptation both for its possible role in understanding protein folding and in designing biocatalysts with enhanced activity at low temperature. The number of reports on enzyme from cold adapted organisms has increased significantly over the past years, revealing that adaptative strategies varies among enzymes. Comparative studies on crystal or model structures and amino acid sequences revealed that each cold adapted enzyme uses different small selections of structural adjustments for gaining increased molecular flexibility that in turn give rise to increased catalytic efficiency and reduced stability. Molecular dynamics simulations provide a powerful tool to understand dynamics of a protein at atomic detail which can lead to significant insights into atomic motions and machinery underlying protein function. In present contribution, we performed molecular dynamics simulations for serine proteases, from mesophilic (*Bos taurus*, *Sus scrofa*) and cold adapted (*Salmo salar*) organisms. The simulations of 6 or 12 ns were carried out in explicit solvent and at two different temperatures, 283 and 310 K, for each protein system. The trajectories were analyzed in terms of secondary structure content, molecular flexibility, intramolecular interactions and protein-solvent interactions. Our analysis highlights how the serine proteases from cold-adapted organisms present more flexible loop regions localized around the active site. Interestingly, these flexible regions show a significantly different amino acid composition between cold-adapted and mesophilic serine protease sequences.

G2-098P
Mammalian copper chaperone Cox17 exists in two metalloforms, linked by oxidative switch

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Cox17, a copper chaperone for cytochrome-c oxidase, is an essential and highly conserved protein from yeast to mammals. Yeast and mammalian Cox17 share six conserved Cys residues, whereas yeast Cox17 has one additional Cys at the N-terminal and mammalian Cox17 has three His residues at the C-terminal

region. Fully reduced mammalian Cox17 binds cooperatively four Cu^{+} ions, whereas partially oxidized Cox17, which contains two disulphide bridges ($Cox17_{2S\text{-}S}$) binds one metal ion, either Cu^{+} or Zn^{2+}. By using X-ray absorption spectroscopy we demonstrate that in Cu_4Cox17 the copper ions on average are coordinated by three sulphur atoms at 2.26 Å and exhibit two specific Cu–Cu distances (2.71 and 2.87 Å), which indicates the presence of a Cu_4S_6-type copper-thiolate cluster in Cu_4Cox17. XAS data for the partly oxidized $Cu_1Cox17_{2S\text{-}S}$ form suggest ligation of the Cu^{+} ion with four light backscattering ligands (N and/or O) at a distance of 1.94 Å and one S at a longer distance of 2.54 Å. pH-stability studies of the two metalloforms of Cox17, studied by ESI MS demonstrate that Cu_4Cox17 is stable at pH values higher than 3.0 whereas $Cu_1Cox17_{2S\text{-}S}$ decomposes already below pH 7.0. Thus Cox17 exists in two different metalloforms containing different metal-binding motifs and oxidative switch is involved in conversion of Cu_4Cox17 to $Cu_1Cox17_{2S\text{-}S}$. This mechanism is capable for simultaneous transfer of up to four metal ions to partner molecules and is suitable for metal transfer to cytochrome-c oxidase and other polycopper oxidases.

G2-099P
Using NMR for a structural proteomics study on the phytopathogen *Xanthomonas axonopodis* pv. *citri*. Structure-based investigation of the function of four unknown proteins

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Xanthomonas axonopodis pv. *citri* (Xac) is the agent of citrus cancer, responsible for substantial economic losses in the production of citrus fruit. Approximately one quarter of Xac genome presents several ORFs encoding proteins of unknown function and/or structure. Since the structural characterization of a protein may provide information regarding its cellular function we have determined the 3D structure of four of those proteins, selected on the basis of a previous NMR assay that assessed their folded state in bacterial lysates. The ORF XAC0862, encoding the protein ApaG, is located in a multifunctional operon that expresses a protein of the pyridoxine biosynthetic pathway, a translational regulatory protein and a diadenosine-tetraphosphate hydrolase. The ORF XAC1516, encodes the protein OmlA. The OmlA genes are often found adjacent to the *Fur* gene, the most important transcriptional regulator of intracellular iron levels. The ORF XACb0070 encodes a protein with low sequence homology with any protein in the PDB. The ORF XAC2000 encodes the protein ClpS that shows 76% similarity with *E. coli* ClpS involved in the recognition of aggregated substrates for the ClpAP proteosome. The proteins were ^{13}C and ^{15}N doubly labelled for NMR studies. Backbone and side chains atoms resonances have been assigned and heteronuclear relaxation experiments have been carried out. The description of the 3D structural features as well of the internal backbone dynamics of these proteins will be discussed aiming to disclose their biological function. Final aim is to discover potential targets for the design of new selective agents against this phytopathogen.

G2-100P
N-terminal propeptide of fungal β-*N*-acetylhexosaminidase plays role in enzyme's folding and dimerization

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β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* is an extracellular enzyme known to be involved in chitin degradation. Sequencing of the enzyme, and cloning of the corresponding *hexA* revealed that the enzyme was produced as a preproprotein, which was processed early in the enzyme's biosynthesis. We found that the propeptide forms a non-covalent complex with the mature protein and it is vital for correct protein architecture and enzymatic function. The processing of the propeptide was essential for activation, dimerization, and secretion of the enzyme. We found that the most frequent form of the extracellular enzyme complex was composed of two molecules of *O*-glycosylated, processed propeptide associated with the mature protein homodimer (composed of two zincin-like domains and two *N*-glycosylated catalytic domains of glycosylhydrolase 20 family). Monomeric catalytic units devoid of propeptide could be detected only as minor intracellular species and they were enzymatically inactive. Moreover, reconstitution experiments with variable amount of propeptide combined with soluble but enzymatically inactive β-*N*-acetylhexosaminidase catalytic subunit allowed us to re-establish the catalytic activity of the enzyme complex. These data strongly suggest that the propeptide plays a crucial role as a "post-translational modulator", facilitating the activation of β-*N*-acetylhexosaminidase.

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G2-101P
"Solid-state proteins" for nanobioelectronic applications

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The use of proteins as electron-conductive materials for bioelectronic/biosensing applications has recently attracted great attention, thanks to the possibility of exploiting their self-assembling capabilities and their specific functionalities at the nanoscale. However, a crucial point is the structural stability of biological macromolecules when used in the solid state and at ambient conditions. In particular, investigations about ageing effects as well as resistance to high electric fields are of fundamental and applicative interest (e.g. biomolecular electronic devices). In this work, we have studied the metalloprotein azurin in both respects. Protein films were maintained at ambient conditions through several weeks, and their conformational changes were investigated by fluorescence spectroscopy. The experimental evidence indicates a weak initial conformational rearrangement, followed by long-term stability. The ageing effects were studied on wild type azurin, as well as on two protein derivatives (apo- and zinc-form): interestingly, the results obtained in the solid state were consis-

tent with the expected structural stability of the three species. Moreover, upon rehydration of the biomolecular films at the end of the investigated period (approximately one month), azurin returns to exhibit a native-like conformation. ''Solid-state proteins'' were also investigated with respect to their resistance to high external electric fields (up to 10^7 V/m) by means of μm-sized interdigitated electrodes. Fluorescence experiments reveal that no significant field-induced conformational alteration occurs. Such results are also discussed and supported by theoretical predictions of the inner protein fields, based on X-ray structural data.

G2-102P
H-bonding in protein hydration revisited

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H-bonding between protein surface polar/charged groups and water is one of the key factors of protein hydration. Here, we introduce an accessible surface area (ASA) model for computationally efficient estimation of a free energy of water–protein H-bonding at any given protein conformation. The free energy of water–protein H-bonds is estimated using empirical formulas describing probabilities of hydrogen bond formation that were derived from molecular dynamics simulations of water molecules at the surface of a small protein, Crambin, from the Abyssinian cabbage (*Crambe abyssinica*) seed. The results suggest that atomic solvation parameters (ASP) widely used in continuum hydration models might be dependent on ASA for polar/charged atoms under consideration. The predictions of the model are found to be in qualitative agreement with the available experimental data on model compounds. This model combines the computational speed of ASA potential, with the high resolution of more sophisticated solvation methods.

G2-103P
Sensibility of two histamine-degrading enzymes to antidepressants in rat and guinea pig tissues

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Histamine is a messenger that is involved in cell to cell communication. This biogenic amine has several physiological effects and is an important mediator in allergy and inflammation. In mammals histamine is mainly degraded by two enzymes: histamine-N-methyltransferase (HNMT) and diamine oxidase (DAO), which are present in most mammal tissues. Enzymatic inactivation pathways of histamine have been suggested to prevent the uptake of ingested histamine from the gastrointestinal tract and to terminate histaminergic signal transmission. Since histamine is an important biochemical molecule, we were interested in the sensibility of histamine degrading enzymes to antidepressants (AD). AD are frequently used drugs in clinical practice. Two types of AD were used in our study: tricyclic antidepressant (TCA) amitriptyline and selective serotonin reuptake inhibitor (SSRI) sertraline. Guinea pig and rat tissue homogenates and plasma have been pre-incubated with different concentrations of AD. A radiometric procedure was utilized to asses HNMT and DAO activity, based on conversion of S-Adenosyl-L-[methyl-14C]methionine and S[14C]putrescine, respectively. Radioactivity was measured by liquid scintillation counting. Our study indicated lower rat plasma DAO activity in the presence of amitryptiline or sertraline, in comparison with the control, whereas guinea-pig plasma DAO activity was higher if antidepressants were added. Rat HNMT activity was higher in the presence of 1–100 μM amitriptyline or 1–50 μM sertraline. Guinea-pig HNMT activity was higher in the presence of 10 nM–1 μM amitriptyline but unchanged in the presence of sertraline. The results indicate that the AD structure is important in influencing the guinea-pig HMNT activity but not in rat HNMT activity or rat and guinea pig DAO activity. It can be concluded that AD can modify the DAO and HNMT activity. The sensibility of histamine-degrading enzymes to AD seems to be species specific.

G2-104P
Self-assembly of peptide nanotubes and amyloid-like structures by charged-termini capped diphenylalanine peptide analogues

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We recently demonstrated that the diphenylalanine core recognition motif of the β-amyloid polypeptide, which is involved in Alzheimer's disease, can self-assemble into a novel class of well ordered peptide nanotubes. We suggested that aromatic interactions contribute order and directionality needed for the formation of these unique nanostructures by such a simple building block. In order to explore the possible role of electrostatic interactions in the assembly process of the peptide based nanotubes we have studied the modified analogue, Ac-Phe-Phe-NH_2, that does not have a net charge. As observed with the NH_2-Phe-Phe-COOH, this analogue self-assembled into highly ordered tubular structures. Another analogue, the peptide NH_2-Phe-Phe-NH_2, which has a net positive charge also self-assembled into tubular structures while amine modified analogues, Boc-Phe-Phe-COOH, Z-Phe-Phe-COOH, Fmoc-Phe-Phe-COOH formed amyloid-like structures. This findings further support our hypothesis regarding the role of aromatic interactions in the self-assembly of amyloid fibrils and amyloid-like nanostructures.

G2-105P
Comparative studies on the structure and stability of fluorescent proteins EGFP, zFP506, mRFP1, ''dimer2'' and DsRed

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Green, yellow and red fluorescent proteins (FP) are widely used as markers of gene expression and protein localization and function in various biological systems. To obtain more information about the structural properties and conformational stabilities of FPs we have undertaken a systematic analysis of series of green and red FPs with different association states. The list of studied proteins includes EGFP (green monomer), zFP506 (green tetramer), mRFP1 (red monomer), ''dimer2'' (red dimer) and DsRed (red tetramer). Fluorescent and absorbance parameters, near-UV and visible CD spectra, the accessibility of the chromophores and tryptophans to acrylamide quenching, and the resistance of these

proteins to the guanidine hydrochloride unfolding and kinetics of the approaching of the unfolding equilibrium have been compared. Green tetramer zFP506 was shown to be dramatically more stable than EGFP, assuming that association might contribute to the protein conformational stability. This assumption is most likely valid even though the sequences of EGFP and zFP506 are only ~25% identical. On the other hand, DsRed was actually shown to be more stable than "dimer2", but mRFP1 was the most stable red FP species. This means that the quaternary structure being an important stabilizing factor doesn't represent the only circumstance dictating the dramatic variations between FPs in their conformational stabilities.

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G2-106P
Porins from Burkholderia pseudomallei and B. thailandensis

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We describe the isolation of two outer membrane porins, *Bps*Omp38 (from *Burkholderia pseudomallei*) and *Bth*Omp38 (from *B. thailandensis*). Both proteins were found to be immunologically related, SDS-resistant, heat-sensitive trimers of M_r ~ 110 000. In functional liposome swelling assays, *Bps*Omp38 and *Bth*Omp38 showed similar permeabilities for small sugar molecules. We also describe cloning and expression of *Bps*Omp38 and *Bth*Omp38 DNAs lacking the signal peptide sequences, using the pET23d(+) expression vector and *E. coli* host strain Origami(DE3). The 38-kDa proteins, expressed as insoluble inclusion bodies, were purified, solubilized in 8 M urea, and then subjected to refolding. As seen on SDS-PAGE, the 38-kDa band completely migrated to ~110 kDa when the purified monomeric proteins were refolded in a buffer containing 10% (w/v) Zwittergent 3-14 with a subsequent heat for 5 min to 95 °C. CD spectroscopy revealed that the refolded proteins contained a predominant beta-sheet structure, which corresponded well to the one of the native Omp38 proteins. The anti-*Bps*Omp38 antibodies considerably exhibited the inhibitory effects on the permeation of small sugars through the Omp38-reconstituted liposomes. A linear relation between relative permeability rates and M_r of neutral sugars and charged antibiotics strongly suggested that the *in vitro* re-assembled Omp38 fully functioned as a diffusion porin.

G2-107P
Localization of respiratory chain complexes in supercomplexes I1III2 and I1III2IV1 from bovine heart mitochondria

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The arrangement of the respiratory chain enzymes in the inner mitochondrial membrane is an issue controversially discussed. According to the Random Collision Model all components (complex I–V as well as ubiquinone and cytochrome c) diffuse separately in the membrane and the electron transport is based on the random collision of the different components involved. The Solid State Model proposes that the respiratory chain enzymes are assembled in so called "supercomplexes" and a direct substrate channelling can take place. Kinetic evidence has been reported recently supporting both models and suggesting a coexistence. Respiratory supercomplexes of different compositions have been isolated from bacteria and mitochondria from yeast, fungi, higher plants and mammals by BN-PAGE and gel filtration. However, no supercomplex could be purified and visualized by structural methods so far. Here, for the first time, we report the purification of supercomplex a (consisting of complex I and III dimer) and supercomplex b (consisting of complex I, III dimer and IV) from bovine heart mitochondria. The functional integrity of the two supercomplexes was demonstrated by activity measurements of all three complexes integrated. Low resolution projection structures of both complexes were obtained by electron microscopy and single particle analysis. A significant structure difference was observed. Complex I, III and IV could be localized within supercomplex b.

G2-108P
Characterization of a b-type variant of *Hydrogenobacter thermophilus* cytochrome c-552 using heteronuclear NMR and MD simulation methods

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Conversion of cytochrome c-552 from Hydrogenobacter thermophilus into a b-type cytochrome by mutagenesis of both haem-binding cysteines to alanines significantly reduces the stability of the protein [1]. To understand the effects of this change on the structure and dynamics of the protein, heteronuclear 15-N-edited NMR techniques [2, 3] and molecular dynamics simulations have been used to characterize this b-type variant. Analysis of the NMR data including chemical shifts, coupling constants, NOE intensities and hydrogen exchange rates demonstrates that the secondary and tertiary structures of the two proteins are closely similar. Some subtle differences are, however, observed in the N-terminal region and in the vicinity of the haem-binding pocket [3]. 8ns MD simulations of the wild-type protein and the b-type variant have been performed at 298 and 360 K. The simulations of the b-type variant show disruptions in the N-terminal helix and greater fluctuations in the contacts between the haem group and protein side chains than those seen in the wild-type protein simulations. Comparisons of the simulations with the experimental NMR data enable the structural and dynamical consequences of removing the thioether linkages to the haem group in a c-type cytochrome to be assessed.

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G2-109P
Thermodynamics of binding some metal ions on human growth hormone

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Human growth hormone (hGH), as a single domain globular protein containing 191 amino acids, plays an important role in somatic growth through its effects on the metabolism of proteins, carbohydrates, and lipids. hGH is produced recombinantly and is available worldwide for clinical use. It has limited stability in solution. Development has therefore focused on more stable and understanding on its interaction with ligands. The interaction of hGH with some of divalent metal ions (Mg, Ca, Co and Cu) in aqueous solution was studied using different techniques. The binding isotherm for hGH-metal ion was obtained by two techniques of potentiometric, using a metal-selective membrane electrode, and isothermal titration calorimetry. There is a set of three identical and non-interacting binding sites for binding of all these metal ions. The intrinsic association equilibrium constants are not very different for Mg (22/mM) and Ca (20/mM), and also their molar enthalpies of binding are similar (–17.5 kJ/mol) showing same thermodynamical properties for hGH upon interaction with both Mg and Ca. The affinity of binding is the highest (116/mM) for Cu and is the lowest (1.25/mM) for Co, but molar enthalpies of binding for both Cu and Co are identical (–16.7 kJ/mol). Thermodynamical properties for hGH upon interaction with Cu and Co not only are different each other, but also completely different from those of Mg and Ca. The circular dichroism spectroscopy study on the protein upon interaction with Cu does not show any changes on the secondary structure of hGH. However, the stability of the protein decreases due to the binding of copper ions.

G2-110P
Hydrophilicity of lysozyme in water–glycerol mixtures

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Different solvents or glassy matrix such as trehalose or glycerol act on proteins as stabilizers, modulating protein's anharmonic motions and the dynamical transition. It can be supposed that these features can be controlled by choosing the physicochemical properties of the protein enviroment and that this perspective would have a number of practical consequences. We performed small angle neutron scattering experiments on lysozyme solvated in glycerol at different water contents to characterize the protein-solvent interface. It was found that the dynamical transition temperature Td of lysozyme powder depends of hydration level h (g water/g Lys). This dependence is very similar if the protein is dissolved in glycerol or not. In particular in both systems an evident decrease of Td occurs at value $0.1 < h < 0.2$. We designed the experiment in order to investigate if the decrease of Td is related to the presence of a water hydration layer that excludes glycerol from protein surface. The samples were prepared at different water contents and deuterium/hydrogen ratio in order to take advantages from contrast variation method. The program used to fit data calculate the scattering curves according to a model that describes the scattering properties of hydration shell, bulk solvent and protein using PDB structure and two free parameters: a thermodinamic constant K and the protein volume. K is the volume concentration of water in hydration shell divided by volume concentration of water in the bulk solvent. The estimated value of $k = 0.6 + 0.2$, obtained from a global fit of 25 scattering curves, demonstrates that glycerol interact with protein surface in every water–glycerol mixture.

G2-111P
Effects of organic solvents on stability of porcine pepsin

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The effects of different concentrations (10–90%) of water-miscible organic solvents [ethanol, tetrahydrofuran (THF), acetone, acetonitrile, dimethyl sulfoxide (DMSO) and N,N-dimethylformamide], and water-immiscible ethyl acetate and toluene on the catalytic activity and structure of pepsin were studied. In ethanol and acetonitrile, the enzyme exhibited high stability; a decrease in activity was observed a concentration of above 60%. At a concentration of 90%, about 30% of the initial activity was preserved. Similar behaviour of the enzyme was observed in THF and DMSO; the enzyme activity decreased continuously with increase of the solvent concentration. The lowest stability was measured in THF; the enzyme activity was practically lost at a THF concentration of 40%. In order to ascertain whether the changes in catalytic activity induced by organic solvents are accompanied by alterations in the secondary and tertiary structures of pepsin, circular dichroism measurements were performed in the far-UV (190–250 nm) and near-UV (250–300 nm) spectral ranges. These studies were carried out in ethanol and acetonitrile. Structurally, pepsin is a monomeric, two-domain, beta-sheet-rich protein. In the presence of higher concentrations of organic solvents, an increase in the alpha-helix content of the enzyme was accompanied by changes in the beta-sheet structure. Our results show that organic solvents cause conformational changes in the pepsin, resulting in a significant reduction in its catalytic activity.

G2-112P
Modelling binding interactions of phosphodiesterase 6 with inhibitors

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Phosphodiesterases (PDEs) are hydrolytic enzymes that decrease the levels of cyclic nucleotides (cGMP and cAMP) in the cell. A retina-specific effector enzyme in the phototransduction cascade, PDE6, hydrolyses cGMP. High-resolution experimental structure of PDE6, however, has not been disclosed as yet. PDE5, a cGMP hydrolyzing isoform, and a close homologue of PDE6, is abundantly expressed in the corpus cavernosum of the male reproductive organ. Inhibitors of PDE5 (Sildenafil, Vardenafil and Tadalafil) are effectively applied in treating erectile dysfunction. Since they are able to cross the blood-retina barrier, concerns have been arisen on possible visual side-effects of these drugs, supported by *in vitro* [1] and *in vivo* [2] data. The first X-ray structures of PDE5 in complex with inhibitors, including sildenafil have been released in 2004. These high-resolution crystal structures provided the opportunity to build a homology model of

PDE6, and to investigate binding interactions between the PDE6 catalytic site and the inhibitors. PDE6 has been modelled in the ligand-bound form using PDE5 as a template. It is found, that sildenafil is able to fit into the catalytic pocket of PDE6 similarly to that of PDE5. This finding is in line with data, that characterize sildenafil with low PDE5 versus PDE6 selectivity [3].

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G2-113P
Evidence of an alternative active conformation of FtsZ at acidic pH

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Several types of bacteria live in highly acidic environment and most of these are pathogenic. The bacterial cell division protein, FtsZ, assembles to form a dynamic "cytokinetic Z-ring" that engineers the bacterial cytokinesis. In addition, FtsZ is well conserved in bacteria. Since assembly of FtsZ is important for bacterial cytokinesis, we examined the effects of pH on the assembly properties of FtsZ and the conformational changes that occur in FtsZ under acidic conditions. FtsZ assembled efficiently at pH 2.5. The rate and extent of FtsZ assembly were found to be similar at pH 2.5 and pH 7. In the presence of divalent calcium, thick bundles of FtsZ protofilaments were formed at both the pHs. In addition, FtsZ polymers displayed similar stability at pH 7 and pH 2.5 indicating that assembly dynamics of FtsZ was not perturbed at pH 2.5. Though FtsZ retained only 25% GTPase activity at pH 2.5 compared to at pH 7, upon refolding from pH 2.5 to pH 7, FtsZ regains its GTPase activity. Interestingly, FtsZ contained similar secondary structures at pH 7 and 2.5 but it had more exposed hydrophobic surfaces at pH 2.5 compared to pH 7. The results suggested that FtsZ attained a molten globule like state at pH 2.5. Further, urea-induced unfolding of FtsZ was studied at pH 2.5 and 7 using multiple spectroscopic techniques and an intermediate state was detected in the unfolding pathways at both the pHs. The Dm values were calculated to be 1.5 and 2.4 M at pH 7 and pH 2.5, respectively, indicating that FtsZ attained a different conformation at pH 2.5 compared to pH 7. The results showed that the functional properties of FtsZ remained intact at pH 2.5, which enable bacteria to perform cytokinesis in acidic environment. Therefore, FtsZ could be a novel target against all pathogenic bacteria as it is a conserved protein in all the bacteria.

G2-114P
Structure determination of the psbH membrane protein in different lipid/detergent environments

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One of the key components for the assembly of Photosystem II is the psbH protein [1]. PsbH is one of the proteins expressed in etiolated and illuminated leaves on the same level in higher plants, which indicates that its function may be considered separately from the rest of the multi-protein complex. The PsbH protein of cyanobacterium *Synechocystis* sp. PCC 6803 was expressed as a fusion protein with glutathione-S transferase (GST) in *E. coli* [2]. We isolated the 15N labeled PsbH protein in concentration of 1.1 mg/ml in presence of the detergent octyl glucoside (OG). We also isolated non-labeled protein for preliminary lipid titration experiments measured by circular dichroism (CD). Molecular dynamics simulations on a homology model of the PsbH protein were carried out and compared to the secondary structure changes observed from CD. The liposomes were prepared by reverse-phase evaporation technique from the thylakoid membrane lipids; sulphoquinovosyl diacylglyceride (SQDG), digalactosyl diglyceride (DGDG), monogalactosyl diglyceride (MGDG) and phosphatidyl glycerol (PG). The most favourable lipid, which induced complex protein folding, detected as formation of the negative band approx. 222 nm in CD spectra, seemed to be PG. Very similar changes were observed at higher concentration also in SQDG, however folding of a clearly different nature was observed upon titration by DGDG. This indicates that protein folding may not be directly related to specific binding of lipids, rather we observe two different types of folding in lipid bilayers of two different properties. The CD measurements revealed folding of the PsbH protein in detergent micelles after addition of sufficient amount of lipid. We added to each protein sample the appropriate amount of lipid to reach optimal protein/lipid ratio. Unfortunately NMR measurements showed a huge decrease of signal and low spectral dispersion of the remaining 15N signals. This is indicative of very rigid lipid-protein micelles, which relax to fast to be recorded. Micelle destabilization using sonication and/or temperature increase led to only partial improvement, therefore we added detergents to the sample; CHAPS and digitonin. Since the mere addition of the detergents did not destabilize micelles sufficiently, we removed the lipids by dialysis. After dialysis the NMR signal was recovered, moreover more dispersed peak positions indicated the presence of a well folded protein. Comparison of the secondary structure content with the molecular dynamics results leads to the conclusion that the combination of digitonin and β-D-octyl-glucopyranoside is the most effective combination to induce stable protein folding.

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G2-115P
Phosphorylation induced preformed structural element in KID contributes to recognition by KIX

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Phosphorylation at Ser-133 of kinase inducible domain of CREB (KID) triggers its binding to the KIX domain of CBP via a concomitant coil-to-helix transition. Although phosphory-

lation is crucial to binding, its exact role is still puzzling: it does not serve as a structural switch between disordered and ordered states, neither its direct interactions account fully for selectivity of KIX for the phosphorylated KID. Hence we reasoned that phosphorylation may shift the conformational preferences of KID in a way to facilitate this binding-coupled folding process. To this end we investigated the intrinsic conformational properties of the unbound KID in phosphorylated and unphosphorylated forms by probing the stability of the bound conformation by molecular dynamics simulations at 310 K and at 600 K. Although no complete unfolding could be achieved, we found that the turn (residues 130–133) linking the two helices and, to a lesser extent, the helices themselves were more resistant to unfolding in pKID than in KID. Complementary free energy calculations show that pSer-133 and Arg-131 of the turn together give –5.2 kcal/mol contribution to the binding free energy that substantially reduce the entropic barrier of folding. Hence we propose that the turn with the highly conserved Arg-Arg-Pro-Ser sequence in KID provides a complementary surface to interact with KIX and functions as a pre-formed structural element. Thus as a recognition motif the turn performs a dual function: (i) it acts as a primary contact site in the process of binding, (ii) it provides a dominant contribution to the free energy of binding via specific interactions with KIX and by restricting the relative orientation of the two helices in KID.

G2-116P
Conformational change of glutamine-binding protein and its complex with glutamine induced by guanidine hydrochloride

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The problem of protein folding to a compact, highly ordered and functional state is one of the central problems of modern structural and cell biology, has also essential practical significance for medicine and biotechnology. The subject of inquiry was glutamine-binding protein (GlnBP), which is responsible for the first step in the active transport of L-glutamine across the cytoplasmic membrane. The aim of this work was examination of unfolding-refolding processes of GlnBP, and examination of complex formation with glutamine on its structure and stability. The equilibrium dependence of tryptophan fluorescence of GlnBP on the concentration of guanidine hydrochloride (GdnHCl) in solution and kinetics of its unfolding by GdnHCl were studied. The fluorescence anisotropy constancy in the range 0–1.0 M GdnHCl for GlnBP and GlnBP/Gln suggests that only at higher concentration of GdnHCl globular structure became disordered. At the same time using fluorescence intensity, fluorescence spectrum position, far- and near-UV CD, ANS fluorescence intensity the existence of two intermediate states was revealed. It is shown that the formation of complex of GlnBP with Gln leads to essential stabilization of macromolecule structure. Nonetheless parametric correlation between fluorescence intensities at 320 and 365 nm shows the existence of two intermediate states in the cause of complex denaturation. It was shown that though GlnBP unfolding is of complex character it is reversible.

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G2-117P
Structure and stability of papain in aqueous organic media

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The effects of different concentrations (10–90%) of ethanol, dimethyl sulfoxide, 1,4-dioxane and tetrahydrofuran (THF) on the stability of papain were studied via intrinsic and extrinsic fluorescence spectroscopic and circular dichroism (CD) measurements. In ethanol and acetonitrile, the intensity of fluorescence of papain (based on Trp residues) increased with increase of the solvent concentration, but in THF and 1,4-dioxane it decreased at high solvent content. In 90% organic solvents, 4–8 nm blue shifts of the maximal intensity values were observed. The enzyme was most stable in ethanol and least stable in THF. In the extrinsic fluorescence measurements, the fluorophore fluorescamine/ 4-phenylspiro[furan-3(3H),1-phtalan]-3,3'-dione/ was used. The binding of fluorophore molecules to papain via primary amines increased with increase of the organic solvent concentration. Far and near- UV CD measurements revealed changes both in the secondary and the tertiary structures of the enzyme. The spectral changes reflect the increase in the ordered secondary structures and a conformational shift towards the beta-sheet structure. Papain has a positive CD spectrum in the spectral range 250–300 nm in water, with a definite fine structure, which originates from aromatic chromophores and intrinsically asymmetric disulfide groups. At 90% solvent content, the low-intensity spectrum completely lacks the bands typical of native papain. The changes in the fluorescence of papain and the results of CD measurements, allow the conclusion of the formation of an intermediate state of the enzyme between the native and unfolded states.

G2-118P
Structural and functional analyses of eukaryotic initiation factor 4B (eIF4B) to study its role in cap-independent translation initiation of picornaviral IRESes

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eIF4B is an eukaryotic initiation factor that helps to promote the association of mRNA with the 40S ribosomal subunit during cap-dependent initiation of protein synthesis. One of the activities of the eIF4B protein is RNA binding and stimulation of the helicase activity of eIF4A. The sequence-specific RNA-binding domain of eIF4B interacts with ribosomal RNA during translation initiation and contains a single RNA recognition motif (RRM). eIF4B is also involved in cap-independent translation initiation driven by picornaviral IRESes. We use structural and functional analyses to probe the role of eIF4B in viral cap-independent initiation in greater detail. The solution

structures of fragments of eIF4B were examined using NMR. Gel shift and filter binding assays were used to determine binding affinity of various eIF4B deletion constructs for EMCV IRES and the *in vitro* A2 RNA. We have already reported the structure of the eIF4B RRM. This fragment in filter binding assays exhibits weak RNA binding affinity to the A2 RNA molecule (KD > 10 μM), but tighter binding was observed for a construct with an extended N-terminus (eIF4B(12–176); KD~0.2 μM). Neither of these constructs bound to the EMCV IRES RNA in filter binding assays. However, we have shown that the protein with an intact N-terminus enhances the RNA binding affinity to both A2 and EMCV IRES RNA. Fragments with C-terminal extensions (1–190, 1–220, 1–250) did not bind with higher affinity to the RNA targets. NMR analyses of these constructs did not exhibit further structure beyond the RRM domain. The core RRM provides only weak interaction with RNA targets and requires an N-terminal extension for higher affinity binding. C-terminal extensions of the fragments do not enhance the strength of the binding.

G2-119P
Effect of d-amino acid substitution on the stability of the MUC2 epitope peptideTPTPTGTQTPT; identification of the degradation fragments by a Q-TOF system

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The development of synthetic, peptide-based immunogens which could undergo processing under specific intracellular circumstances, but are stable before reaching the targeted site would be important for more efficient immunization. In order to investigate the effect of structural modification (D-amino acid substitution) on enzymatic stability epitope 15TPTPTGTQTPT25 from the tandem repeat unit of mucin 2 glycoprotein (MUC2) was chosen. The peptide is recognized by protein specific antibodies even in tumour tissue. Different number of amino acids at the C- and/or N-terminal were replaced by the D-analogue. The sequence responsible for the immunoresponse is in the centre of the peptide (PTGTQ), while the D-amino acids are in the flanking regions of the N- and/or C-terminal. The stability of these peptides was studied in human serum at two concentrations (10% and 50%) and in a lysosomal preparation of rat liver (at pH = 3.5 and 5.0). The enzymatic degradation was followed by RP-HPLC method. Our intention was to identify the degradation fragments by their mass, as well as to determine the effect of the quantity and the position of the D-amino acids on the degree of degradation. Molecular weights were identified by a Q-TOF system. We found that the D-amino acid substitution had a different effect on the stability of peptides in human serum and in lysosome homogenate: in lysosome homogenate the peptides containing D-amino acids only at the C-terminal are already stable, while in human serum the substitution at both terminal is necessary for the complete stability. Depending on the quantity of the D-amino acids the formation of the different fragments changes either in lysosome homogenate or in human serum. Considering our results we can say that the incorporation of D-amino acids in the MUC2 epitope peptide increase completely their stability which was proved by both RP-HPLC and mass spectrometry.

G2-120P
NO-binding properties and (non-)enzymatic reduction of human neuroglobin

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The function of neuroglobin (Ngb), a vertebrate globin, is yet unknown. Several possibilities can be suggested, amongst which an involvement in the NO metabolism, a possible oxygen-carrier function under hypoxic conditions or even a cytochrome-like redox function. Electron paramagnetic resonance (EPR) and optical measurements show that, both the ferrous and ferric forms of wild-type Ngb are found to be hexacoordinated with axial ligation of the F8-His and E7-His. When wild-type human Ngb (wt NGB) is overexpressed in *E. coli* cell cultures with low O_2 concentration, the protein is predominantly in the F8His-Fe2+-E7His form, whereby a small percentage of the protein is in the nitrosyl ferrous form. In contrast, analogous studies for mutated NGB (E7-His to Leu and Gln) reveal the predominant presence of the nitrosyl ferrous form. The EPR spectra of the NO-ligated NGB proteins consist of contributions from two geometrically different NO-heme conformations. The temperature dependence of these spectra proves a strong stabilization of one isomer by the E7-His in wt NGB. The non-enzymatic reduction of the ferric form of wt NGB with NAD(P)H is very slow and is biologically irrelevant. When the cell lysates of *E. coli* cells overexpressing wt NGB are kept under oxygen, the oxidation of NGB is very slow in contrast to the fast autoxidation rate of the protein. If the lysate is partitioned in a high- (HM) and low-molecular (LM) fraction, NGB in the HM oxidizes rapidly to the ferric form. However, when the HM and LM fraction are recombined, a rapid reduction of the iron occurs, indicating the presence of a reducing enzymatic system in the lysate. A similar reduction occurs when NAD(P)H is added to the HM fraction. The biological relevance of all experiments will be discussed.

G2-121P
Cooperativity and site-selectivity in human ileal bile acid-binding protein

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Human ileal bile acid-binding protein (I-BABP) is expressed in the absorptive cells of the distal small intestine and thought to function in the enterohepatic circulation of bile salts. Bile salts are amphipathic steroids synthesized from cholesterol in the liver. In the GI tract they act as detergents that aid in the absorption of dietary lipids. Three major bile acids (cholic acid, chenodeoxycholic acid, and deoxycholic acid) in the form of glycine or taurine conjugates represent greater than 95% of all bile salts in humans. I-BABP binds two molecules of glycocholate with weak intrinsic affinity but an extraordinary high degree of positive cooperativity as determined by isothermal titration calorimetry

and an NMR based site-specific binding assay. The NMR structure of the ternary complex of I-BABP with two different bile salts, glycocholate (GCA) and glycochenodeoxycholate (GCDA), has defined the structural topology of the doubly ligated I-BABP and revealed a number of potential protein/ligand contacts specific for each binding site. To understand the role of these contacts we have investigated the energetics of binding of GCA and GCDA to a number of engineered I-BABP mutants. To gain further insight into the structural correlates of binding cooperativity we are currently solving the NMR structure of a doubly ligated mutant I-BABP that exhibits diminished cooperativity in both GCA and GCDA binding. The experimental strategies include: (i) isotope-edited/filtered NOESY experiments using C-13/N-15 enriched protein and unenriched bile salts, and (ii) isotope-edited NOESY experiments using selectively C-13 or N-15 enriched bile salts and unenriched protein. Comparison to the structure of wild-type protein will be presented.

G2-122P
Studies on the epitopes of Malayan pit viper venom hemorrhagin

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The continuous epitopes of the major hemorrhagin (a metalloproteinase) isolated from Malayan pit viper (*Calloselasma rhodostoma*) venom were located by examining the cross-reactions between polyclonal antibodies against the hemorrhagin and peptides derived from endoproteinase Lys-C and Glu-C digestion, using dot-blot immunostaining technique. Three antigenic sites were located: peptide E-8 (residue 111–120), E-3 (165–177) and K-21 (46–76). Predicted secondary structure of the hemorrhagin indicates that these three peptides correspond to "turns", which are common features of antigenic sites. Comparison of the Hopp-Wood hydrophilicity profile with the experimental results indicated that the oligosaccharide moieties of the hemorrhagin do not function as antigenic determinants. Comparison of the Hopp-Wood hydrophilicity profile of the hemorrhagin with those of other snake venom metalloproteinases indicates that although the metalloproteinases share common epitopes, the epitopes shared are probably not the same in all the metalloproteinases. Knowledge of these common antigenic sites would provide useful data for the synthesis of peptides which could be used as synthetic antigens for the production of anti-hemorrhagin antibodies.

G2-123P
Universal and sequence-specific behaviour in protein misfolding and aggregation

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A range of unrelated human diseases such as Alzheimer's, spongiform encephalopathies and light-chain amyloidosis lead to degenerative conditions and involve the deposition of plaque-like material in tissue arising from the aggregation of proteins. In all cases one observes a transition from the proper native structure, which might be rich in α-helices, to β-strand rich structures which favors aggregation and causes disease, known as amyloid fibrils. Increasing evidence is accumulating that almost all kind of such fibrils are characterized by the same parallel in-register aggregation between identical sequence portion of different chains in the aggregate. We show that in-register aggregation can be explained in a very simple manner by deriving an energy function, based on the knowledge of protein native structures from the Protein Data Bank, which takes into account the propensity for a given residue pair to be hydrogen bonded in neighbouring β-strands. We then use the same energy function to predict the minimal peptide fragment which is most likely to drive the formation of amyloid fibrils, comparing successfully our results with known experimental data in a variety of cases, comprising β-amyloid peptide, α-synuclein, islet amyloid polypeptide. Such results show that there is a unifying theme in the sequence-specific properties of aggregation in amyloid fibrils. We then suggest, on the basis of computer simulation of a simplified model, that the common attributes of all proteins, and not the details of the amino acid sequence, sculpt the free energy landscape and determine the fixed menu of putative native state folds and that the general propensity for amyloid formation is also a direct outcome of the same common features of all proteins.

G2-124P
High resolution crystal structures of HIV-1 protease mutants with potent new inhibitors

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Atomic resolution structures of the HIV protease with a new clinical inhibitor UIC-94017 (TMC114) are described. Most of the problems associated with the HIV therapy, such as rapid development of drug-resistant variants and side effects of drugs, are consequences of the necessarily long-term use of the drugs. The new inhibitor has high potency and excellent resistance profile. It is designed to induce tighter inhibitor interactions with the protease, and thus reduce the resistance caused by mutation. These high resolution crystal structures (1.6–1.1 Å) have proved that the inhibitor design goal has been met and will guide the design of potential drugs. Also, the inhibitor has been studied with frequently observed resistant mutants of HIV-1 protease (I50V, D30N, V82A and I84V) in order to define any structural changes associated with mutations. The new crystal structures will be important for the design of the next generation of HIV protease inhibitors which will more effectively target resistant virus.

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G2-125P
Conformational stability and structural dynamics of membrane-bound alkaline phosphatase from *Echinococcus granulosus* metacestode during urea-induced unfolding process

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Hydatid disease, caused by the larval stage (metacestode) of *Echinococcus granulosus* (cestoda), is a world-wide public health problem. The membrane-bound alkaline phosphatase, has a key role in metacestode development and host-parasite interface. In the present study, conformational stability of alkaline phophatase from membranes of hydatid cyst was identified by unfolding process with the chemical denaturant, urea. After *n*-butanol extraction, ALP was purified from hydatid cyst membranes by affinity chromatography on concanavaline A-Sepharose column followed by gel filtration. Purified enzyme was incubated with various concentrations of urea in Tris-acetate buffer at 30 °C for 24 h. The process of enzyme unfolding was monitored by assaying enzyme activity and fluorescent spectroscopy. The unfolding curve of enzyme activity change followed a three-state unfolding model. The Michaeilis constant for substrate, 4-nitrophenyl phosphate, was not change at all in urea concentration tested. In fluorescence spectroscopy process, ALP showed five unfolding states induced by urea. The [urea]0.5 levels corresponding to each unfolding intermediates were 3.1, 4.6, 5.3, 6.5 and 7.4, respectively. One of the urea induced unfolding intermediates was stabilized by addition of guanidinium chloride but this multiphasic phenomenon changed to monophasic by adding NaCl. Multiple unfolding states of ALP induced by urea implied differential stability of the subdomains of the enzyme. It was suggested that the substrate-binding region of the enzyme is a more constant structure in chemical denaturing than the other structural domains. The data for fluorescence quenching experiments and structural dynamics of ALP during urea unfolding will be discussed.

G2-126P
Enzyme-substrate interaction through docking simulation – Part I. Structure analysis of angiotensin-I converting enzyme complexes with various peptides

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Effective regulation of hypertension is still a major research challenge since cardiovascular disease is the leading cause of death in the so-called developed countries. Many lethal cardiovascular events are due to the failure to reduce blood pressure in hypertensive individuals. The angiotensin-I converting enzyme (ACE) is a zinc metallopeptidase and it is a major component of the rennin–angiotensin–aldosterone system (RAAS). This system together with certain metallopeptidases, plays a central role into metabolism of peptide hormones crucial for blood pressure modulation. ACE and other gluzincin peptidases, such as neutral endopeptidase (NEP), and endothelin converting enzyme (ECE), metabolize various peptides, like angiotensin I and II (AI/AII), bradykinin (BK), luteinizing hormone releasing hormone (LHRH), atrial natriuretic peptide (ANP) and endothelins (ETs), which are essential for arterial tone and water-electrolyte homeostasis regulation. Simulation of ACE-peptide complexes using recently determined ACE [1] and NEP [2]. X-ray structure and peptides NMR models through simulating annealing or genetic algorithms could elucidate the structure and physicochemical determinants govern the enzyme-substrate affinity and specificity. For this purpose the NMR structure of ACE testis X-ray structure and the solution model of angiotensin-I, bradykinin and LHRH are implemented in AUTODock software and results yielding ACE-substrate interaction model with favourable geometry and low binding free energy. Such an approach is currently applied in our laboratory in order to extract valuable insight for structure-based design of new bioactive compounds.

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G2-127P
An easy optimalization method for *in vitro* protein refolding – the case of CD69

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In protein biochemistry we must often solve a problem of renaturation (refolding) of proteins of our interest. Especially in the case of bacterial recombinant proteins, which are often obtained in insoluble form (the inclusion bodies), we must find an optimal way to transfer the protein molecules from denaturating agents to native conditions. We use an easy optimalization method for *in vitro* refolding of such proteins, which is critical and demanding task. Successful renaturation of the protein is a multiparameter optimalization procedure that involves finding of certain discrete conditions, such as presence of suitable additives (i.e. arginin), pH, ionic strength and redox potential of the system. These parameters are optimalized in semimicroquantitative way. Here we present results for CD69 protein [1], an early activation antigen of human lymphocytes. Efficiency of the refolding was analyzed by gel filtration and SDS-PAGE. Secondary structure of receptor CD69 prepared via such optimalized protocol was determined by drop coating deposition Raman spectroscopy method [2].

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G2-128P
Relationship between the in-plane asymmetry of the heme electronic structure and axial methionine coordination in cytochrome *c*

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Functional properties of metalloproteins depend crucially upon the electronic nature of metal ions, which is regulated through the interaction with the polypeptide chains. Paramagnetic metalloproteins take advantage of solution NMR studies, providing a wealth of unique information on the electronic structure of the active sites, which can be directly correlated with the functional properties [1]. We have shown previously that the interaction of lone pair of the axial methionine sulfur atom with the d_π (d_{xz} and d_{yz}) orbitals of heme iron is largely responsible for the unpaired electron delocalization in the porphyrin π system of heme in the oxidized form of cyt *c*, and hence that axial methionine coordination is a major determinant for the in-plane asymmetry of the heme electronic structures within the scaffold of the protein folding in cyts *c* [2]. In the present study, we have extended our exploration toward discovering structural factors responsible for the control of the heme electronic structure, using homologous *Pseudomonas aeruginosa* cytochrome c_{551} (*PA*) and *Hydrogenobacter thermophilus* cytochrome c_{552} (*HT*) and a series of their mutants, i.e. *PA* N64Q, *PA* N64A, *HT* Q64N, and *HT* Q64A. The study not only confirmed that the heme electronic structure in cyt *c* is predominantly determined by the axial methionine coordination structure, but also revealed that the methionine coordination is regulated by local interaction with nearby amino acid residues.

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G2-129P
Interactions of ceruloplasmin and dietary polyphenols in expressing of their total antioxidant activity towards $ABTS^{\bullet+}$

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Ceruloplasmin (CP, ferro-O_2-oxidoreductase, EC 1.16.3.1) is an antioxidant of human plasma. Polyphenols are important antioxidants of plant origin, which are one of major human nutrition antioxidants. There is limited data regarding the accurate estimation of polyphenolics of plant origin intake and the retention of their active metabolites in plasma. Concentrations of plasma antioxidants (vitamins E and C, uric acid) were shown not be influenced by the consumption of dietary polyphenolics, but there is no data concerning CP. Therefore, the aim of this study was to investigate interactions of CP with polyphenols in expressing of their total antioxidant activity (AA). Gallic, ferulic, sinapic and protocatechuic acids, (±)catechin, (–)epicatechin and human CP were used in this study. AA was determined by ABTS (2,2'-azinobis(3-ethilbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay in kinetic mode. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with ammonium persulfate and reaction conditions were optimized for 24 h stability of radicals produced. Kinetic of $ABTS^{\bullet+}$ decay in presence of CP and mixture of CP with individual polyphenols was monitored for a duration of 6 min at 734 nm. The results obtained draw to several conclusions: AA towards $ABTS^{\bullet+}$ of CP in mixture of polyphenols tested were at the same level as summarized AA of individual polyphenols and CP. Nevertheless kinetic curves were significantly different reflecting complex character of CP and individual polyphenols interaction. The kinetic parameters for CP, system of CP and individual polyphenols have been calculated. The kinetic mechanism of AA expressing by mixture of CP and polyphenols has been proposed.

G2-131P
Structural characterization of cyclase-associated protein (CAP) domains

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Cyclase-associated protein (CAP) is a highly conserved and widely distributed protein required for normal cell growth and development. In yeast, CAP is a component of the adenylyl cyclase complex and helps to activate the Ras-mediated catalytic cycle of the cyclase. Full-length CAP constitutes an N-terminal domain (N-CAP), required for Ras response, a C-terminal domain (C-CAP), that interacts with the cytoskeleton, and a proline-rich middle domain, which can bind to SH3 domains. N-CAP and C-CAP have been shown to be dimeric in their crystal structures [1, 2]. Our own crystallographic studies on N-CAP from *Dictyostelium discoideum* [3, 4] have revealed the presence of both a head-to-tail dimer conformation, along with the previously reported side-to-side dimer [1]. These interactions are indicative of the variable modes of oligomerization, which makes studies on full-length CAP difficult to perform. We present the results of experiments to elucidate the oligomerization behaviour of CAP in solution and their comparison to findings from the crystal structures.

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G2-132P
The relative stability of homochiral and heterochiral alanine dipeptides. Effects of perturbation pathways and force-field parameters on free energy calculations

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The relative stability of homochiral (D,D or L,L) and heterochiral (D,L or L,D) dipeptides may have been a decisive factor in the evolutionary propagation of a symmetry-breaking event leading to the present-day predominance of L-amino acids in natural proteins. Kinetic resolution in the solid-phase peptide synthesis of blocked dialanine suggests the activation free energy difference of formation of (D,D or L,L)- and (D,L or L,D)-dialanine to be 0.22 kJ/mol in favour of the formation of the homochiral dipeptide. Computer simulation studies were performed on water-solvated dialanine, applying a thermodynamic integration protocol using the GROMOS force field. Five different pathways and three force-field parameter sets have been used to assess the possibility of a computational prediction of the chiral preference. Inversion of the configuration around either one of the Cα-atoms by changing the improper dihedral angle, while the bond angles undergo a temporary opening, leads to an excellent reproduction of the experimental result.

G2-133P
Modification with fluorescent labels desensitizes muscle FBPase toward calcium inhibition

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Fructose-1,6-bisphosphatase (FBPase) [EC 3.1.3.11] catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and Pi in the presence of divalent cations like magnesium, manganese, zinc or cobalt. Liver and muscle FBPase isozymes have been found in mammalian tissues. Both isozymes are activated by monovalent cations like potassium or ammonium, inhibited competitively by fructose-2,6-bisphosphate and allosterically by AMP. The liver isozyme has been recognized as the key enzyme of gluconeogenesis, the muscle isozyme as the key enzyme of glyconeogenesis. The basic difference between muscle and liver isozymes concerns their sensitivity to AMP inhibition. $I_{0.5}$ of the muscle isozyme is 50–100 times lower than the corresponding value for the liver isozyme. Recently we have found that also calcium is a strong inhibitor of muscle FBPase [1] with K_i = 590 nM. Searching for the possible mechanism of the enzyme inhibition by calcium we found that modification of muscle FBPase with rodamine- (TRITC) and fluorescein-isothiocyanate (FITC) results in the significant decrease of the enzyme sensitivity against calcium inhibition (K_i is 350 times increased) and slight desensitization against inhibition by AMP ($I_{0.5}$ is 10 times increased). On the other hand, K_m and K_i for the competitive inhibitor are unchanged. Digestion of muscle and liver FBPase with subtilisin results in cleavage of N-terminal peptide, shift of pH optimum into alkaline region and decrease of enzyme sensitivity toward AMP inhibition. We found that digestion of muscle FBPase with subtilisin results in loss of the enzyme sensitivity also against calcium inhibition. Further study are in progress to determine the calcium binding site in muscle FBPase.

Reference

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G2-134P
Spontaneous deamidation of the *Bacillus anthracis* protective antigen impairs its cleavage efficiency by furin

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Protective antigen (PA) protein, purified to apparent homogeneity from *Bacillus anthracis* growth medium, displays reduced *in vitro* J774 macrophages cytotoxicity ($T_{½} \approx 7$ days) under storage conditions at 25 °C (pH 8.0). This reduced activity was followed by a concomitant increased number of acidic isoforms, suggesting the involvement of a deamidation process. Indeed, by a combination of limited tryptic cleavage, SDS-PAGE and AspN in-gel digestion, followed by MALDI-TOF/MS analysis, it is shown that PA undergoes spontaneous deamidation at Asn_{162} with a gradual decreased susceptibility to its essential activator protease-furin. A series of synthetic peptide substrates, encompassing the cleavage site of PA by furin were used to demonstrate that deamidation at Asn_{162} results in a 10-fold decrease in the cleavage efficiency of the peptide substrate by furin. This reduced efficiency is due to the appearance of a new negative charge at position P_6 of the substrate, and to a lesser extent by the insertion of an additional $–CH_2–$ group in the peptide backbone.

G3–Protein-Protein Recognition

G3–001
Prediction of protein-protein and protein-ligand interactions from protein structures

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World-wide structural genomics initiatives are rapidly accumulating structures for which limited functional information is available. Additionally, state-of-the art structural prediction programs are now capable of generating at least low-resolution structural models of target proteins. Accurate detection and classification of functional sites (protein-protein and protein-ligand interaction sites) within both solved and modelled protein structures therefore represents an important challenge. A fully automatic site detection method, FuncSite, that uses neural network classifiers to predict the location and type of functionally important sites in protein structures will be discussed. The method is designed primarily to require only relative residue position without the need for specific side-chain atoms to be present. The functional site

encoding represents conservation using PSI-BLAST PSSMs of site residues as well as solvent accessibility and secondary structure assignments. We have rigorously benchmarked FuncSite on a set of metal binding sites spanning numerous SCOP super-families. The method has also been extended to the prediction of protein-DNA interface regions, adenylate classification and the identification of enzyme active sites. In addition a prototype method for inferring an interaction footprint between two protein domains based on the optimization of contact potentials and a shape complementarity function will also be outlined and preliminary results discussed.

G3–002
Structural basis of glycolytic compartmentation: a cytoarchitect's guide to metabolism

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Compartmentation of carbohydrate metabolism (metabolite channeling) has been shown in a wide range of tissues. Although the enzymes for glycolysis (GLY), glycogenolysis, gluconeogenesis (GNG) are found in the cytoplasm, elements of the cytoarchitecture may organize metabolism to meet localized energetic demand. In smooth muscle we found that vinblastine and taxol additively decreased GLY flux but did not alter GNG flux determined by 13C-NMR. Those results were consistent with a localization of GLY enzymes with microtubules (MT) and two separate MT binding sites for GLY enzymes. We have demonstrated that GLY and GNG are compartmented and hypothesized that two plasma membrane domains were responsible. Although plasma membrane compartments have been described, only in the erythrocyte has the physical basis for plasma membrane associated glycolytic pathway been established. We found that caveolin-1 (CAV-1) may serve as a scaffolding for GLY enzyme localization since phosphofructokinase (PFK) appears to colocalize with CAV-1 at the cell membrane (by confocal immunofluorescence). Cell knock-down of CAV-1 with siRNA reduced CAV-1 and PFK membrane localization and colocalization. Overexpression of CAV-1 in smooth muscle increased PFK membrane localization and colocalization of PFK and cav-1. Expression of CAV-1 in lymphocytes (which normally do not express CAV-1) altered PFK localization from uniformly cytoplasmic in control lymphocytes to almost exclusively membrane localized in transfected lymphocytes. We conclude that CAV-1 may function as a scaffolding protein for PFK. From these studies a picture is emerging that cytoplasmic enzyme systems may be organized by a variety of elements of the cytoarchitecture.

G3–003
The role of autophagy in degradation of intracellular protein aggregates

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CNS neurons are endowed with the ability to recover from cytotoxic insults associated with the accumulation of proteinaceous polyglutamine aggregates in Huntington's disease, but the cellular mechanism underlying this phenomenon is unknown. Here we show that autophagy is essential for the elimination of aggregated forms of mutant huntingtin. Autophagy is considered a highly regulated but non-selective pathway by which cytoplasmic constituents are degraded in lysosomes in response to nutrient deprivation. Our data show that autophagy is induced in response to impaired activity of the ubiquitin proteasome system. Autophagins, molecular determinants of autophagic vacuole formation, are recruited to aggresomes, pericentriolar cytoplasmic inclusion bodies that form when the capacity of the ubiquitin proteasome system is exceeded. Inhibition of autophagy with RNA interference demonstrates that this pathway is essential for cells to eliminate aggregated huntingtin. Recruitment of autophagins to aggresomes and aggregate clearance both require an intact microtubule cytoskeleton, suggesting that retrograde transport on microtubules is a mechanism used by cells to increase the efficiency and selectivity of this degradation pathway. Thus, autophagy is key component of the cellular defense against protein aggregation.

G3–004
A human protein atlas for proteome profiling in normal and disease tissue

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The Swedish Human Proteome Resource (HPR) program, funded by the Knut and Alice Wallenberg Foundation, has been set-up to allow the systematic exploration of the human proteome with Affinity (Antibody) Proteomics, combining high-throughput generation of affinity-purified (mono-specific) antibodies with protein profiling using tissue arrays. The basic concept of the resource centre is to produce specific antibodies to human target proteins using a high-throughput method involving the cloning and protein expression of Protein Epitope Signature Tags (PrESTs). The antibodies are subsequently used for functional analysis of the corresponding proteins in a wide range of assay platforms. See also http://www.hpr.se.

G3–005
Modulation of prion formation, aggregation and toxicity by cytoskeletal proteins of the vesicle assembly complex in yeast

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Prions are protein isoforms that cause transmissible neurodegenerative diseases in mammals and control heritable traits in fungi. Most of the known prions are self-perpetuating amyloid-like ordered fibrous protein aggregates, that propagate prion state by immobilizing the soluble protein molecules of the same amino acid sequence. Yeast prion [PSI+] is an aggregate of the translation termination factor Sup35. Prion domain of Sup35 is rich in glutamine (Q) and asparagine (N) residues, thus resembling poly-Q proteins, such as huntingtin, which is involved in Huntington's disease. While recent data shed light on the major steps of propagation of the pre-existing [PSI+] aggregates in the yeast cells, mechanisms of initial formation of a prion from non-prion protein remain a mystery. De novo formation of the [PSI+] prion is induced by transient overproduction of Sup35 protein or its prion domain. We demonstrate that prion domain of Sup35 interacts with various components of the actin assembly complex that is involved in generation of endocytic vesicles. Alterations of the components of the vesicle assembly complex decrease aggregation of overproduced Sup35 and de novo prion

formation, and increase toxicity of overproduced Sup35 in the yeast cells. Depletion of the cytoskeletal protein Sla2, which is homologous to the mammalian huntingtin-interacting protein Hip1, is synthetically lethal with the prion form of Sup35. Our data show that cortical cytoskeletal networks are involved in generation of prion aggregates, and suggest that prion formation may play a certain protective role in the conditions of protein overproduction.

G3–006
Universal method for synthesis of highly selective artificial gel antibodies against proteins, viruses and cells; some techniques to study the selectivity and applications

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Highly selective artificial gel antibodies against different classes of antigens can be prepared by an imprinting method, which thus, is universal: An aqueous solution of the antigen is supplemented with appropriate monomers. Following polymerization the gel is granulated and the antigen is removed. The degree of selectivity can be determined by various techniques. We will pay special attention to free zone electrophoresis in a rotating capillary and QCM (Quartz Crystal Microbalance). In the former method the mobility of the complex between the gel antibody and the antigen present during the polymerization is determined, as well as the mobilities of complexes between this gel antibody and antigens which are structurally related to the above antigen. The combination of this synthesis of artificial gel antibodies with analysis by free zone electrophoresis permits detection of extremely small differences in the structure of biomolecules and bioparticles, which will be demonstrated by several examples (for instance, we can differentiate between different strains of bacteria, between wild type and a mutant of virus, between proteins from different species, etc.). The gel antibodies have many advantages compared to those raised in experimental animals (an ethical problem): more stable, probably more selective, more cost-effective to produce, and they can be synthesized not only against proteins, but also against particles, such as viruses and cells, for instance bacteria (in animals these particles are degraded metabolically). We will discuss some of the many potential applications, based on these gel antibodies, in order to stimulate the participants of the meeting to employ them as flexible tools in their own projects.

G3–007P
Co-immunoprecipitation and fret between c-FOS and enzymes of phospholipid sythesis it activates

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We have previously demonstrated that the protooncoprotein c-Fos has the capacity to associate to the endoplasmic reticulum (ER) and activate phospholipid biosynthesis (PLB) necessary for the genesis of membrane required for cell growth. This activation is independent of its transcription factor capacity (Faseb J, 2001: **15**;556, *Mol Biol Cell,* 2004: **15**;1881). Using c-Fos deletion mutants, we determined that the protein domain called BD of c-Fos (aa 139-159) is relevant for this activation: only mutants containing BD associate to the ER and activate PLB. Herein, we examined a possible direct interaction between c-Fos and some of the enzymes it activates. For this, pcDNA3.1myc was used to express Phosphatidylinositol synthase (PIS1), CDP-DAG synthase (CDS1), CDP-choline cytidylyltransferase (CPT), Choline transferase (CT) and the alpha and beta subunits of the kinase PI4KII in NIH 3T3 cells. PI4KII, CPT and CT co-immunoprecipitate with c-Fos as determined by Western blot analysis, indicating a direct interaction between the enzymes and c-Fos. To confirm this, we measured FRET (Fluorescence Resonance Energy Transfer) on a confocal microscope between PIS1, the alpha or beta subunits of PI4KII fused to the CFP protein and c-Fos fused to YFP protein, expressed in NIH 3T3 cells. When measured as percentage of efficiency of FRET, only the alpha subunit, but not the beta subunit of PI4KII, was found interacting with c-Fos, in agreement with the results obtained by co-immunoprecipitation assays. These results strongly support that a directinteraction between c-Fos and the PLB enzymes that it regulates is required for its cytoplasmic activity.

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G3–008P
Molecular characterization of the 5-HT4 receptor dimerization process

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Serotonin 5-HT4 receptors (5-HT4R) are members of the family I of G protein-coupled receptors (GPCRs) and molecular studies have revealed the existence of several splice variants, which differ in their C-terminal tails. Given the recent recognition that many GPCRs can dimerize, the present study was undertaken to determine whether 5-HT4R can form dimers and what are the molecular determinants involved in the dimerization process. Using co-immunoprecipitation and bioluminescence resonance energy transfer (BRET), we showed that the human 5-HT4(d)R isoform can form homodimers when expressed in CHO and HEK cells. Selective 5-HT4R ligands did not influence the degree of 5-HT4(d)R dimerization process. We also found that co-expression of h5-HT4(d)R and h5-HT4(e)R isoforms led to a constitutive energy transfer, providing the first evidence that 5-HT4R can form heterodimers in living cells. In addition, we showed heterodimer formation between the 5-HT4(d)R and the beta2-adrenergic receptor. Basal BRET signal was not influenced by 5-HT4 and beta-adrenergic ligands. Interestingly, co-immunoprecipitation and BRET experiments revealed that the reducing agent dithiothreitol (DTT) inhibited 5-HT4(d)R dimer formation and shifted the receptor into its monomeric form suggesting that disulfide bridges are specifically involved in the formation of 5-HT4R dimers. To test this hypothesis, we localized and mutated cysteines potentially involved in 5-HT4(d)R dimerization interface using GRAMM computer algorithm and site-directed mutagenesis. Surprisingly, we report that two cysteines located in the 3rd and the 4th transmembrane domains are critical for the stability of the 5-HT4R dimer complex.

G3–009P
The CDR3 regions of an immunodominant T cell receptor dictate the 'energetic landscape' of peptide-MHC recognition

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The energetic bases of T cell recognition are unclear. Here, we studied the "energetic landscape" of peptide-major histocompatibility complex (pMHC) recognition by an immunodominant T cell receptor (TCR). We quantified and evaluated the effect of natural and systematic substitutions in the complementarity-determining region (CDR) loops on ligand binding in the context of the structural detail of each component of the immunodominant TCR-pMHC complex. The CDR1 and CDR2 loops contributed minimal energy through direct recognition of the antigen and instead had a chief function in stabilizing the ligated CDR3 loops. The underlying energetic basis for recognition lay in the CDR3 loops. Therefore the energetic burden of the CDR loops in the TCR-pMHC interaction is variable among TCRs, reflecting the inherent adaptability of the TCR in ligating different ligands.

G3–010P
Glycolytic enzymes are bound to yeast mitochondrial surface: proteomic study.

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Traditionally, glycolytic enzymes are considered as typically cytoplasmic proteins. Studying yeast glycolytic enzyme enolase 2 (one of two functional enolases in the yeast cell), we found that it participates in tRNA import into mitochondria and is partially associated with mitochondrial outer membrane (MOM). The last was proven by confocal microscopy, Western-blot analysis of mitochondrial subfractions and assays of protein import into isolated mitochondria. It was demonstrated that 7 of 10 glycolytic enzymes were associated with MOM in *Arabidopsis* (P.Giege et al., 2003). We measured activity of all glycolytic enzymes associated with surface of mitochondria from *Saccharomyces cerevisiae*, human cells HepG2 and bovine liver. It was shown that at least 5 enzymes were partially bound to MOM. We analyzed MOM-associated complexes containing enolase by two proteomic approaches: 2D -Blue Native PAGE and co-immunoprecipitation followed by MALDI-TOF. Results obtained by both methods were consistent and supplemented each other. We identified enolase-containing macromolecular complex, included two isoenzymes of glyceraldehyde-3-phosphate dehydrogenases, pyruvate kinase 1, mitochondrial transport proteins and some others proteins involved in cell metabolism. These data are in agreement with the hypothesis that glycolytic enzymes might be physically associated with one another to facilitate substrate channelling on the surface of yeast mitochondria. This work was supported by RFBR, CNRS, MitEuro (EU Contract No. QLG1-CT-2001-00966). 1. Giege P, Heazlewood JL, Roessner-Tunali U, Millar AH, Fernie AR, Leaver CJ, Sweetlove LJ. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells.

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G3–011P
Nuclear translocation of hypoxia-sensing transcription factors: involvement of the importin alpha/beta-system

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HIF (hypoxia inducible factor) family members act as transcriptional master regulators of genes involved in cellular and systemic oxygen homeostasis. HIF-1 is the prototypical member of the family and the most important factor in the cellular response to hypoxia. HIF-1 consists of two subunits HIF-1a and HIF-1b. While the different factors concerned in regulation and stability of HIF-1a are described in detail, the knowledge of the translocation process is still imprecise. The dimerization of the subunits takes place in the nucleus and is necessary for DNA binding and transcriptional activation of target genes like erythropoietin or glucose transporter 1. Nuclear import occurs through the nuclear pore complex (NPC). Therefore, passing the NPC is one of the major events in the HIF-1 dependent transactivation process. Small molecules up to 60 kDa may passively diffuse into the nucleus, whereas larger substrates, like the Hypoxia-Inducible-Factors are restrained. Import of macromolecules into the cell nucleus is mediated by soluble import factors, which bind their import substrates at specific nuclear localization signal sequences (NLS). This implies that the specific signal sequences must exist in the imported molecules to mediate the transport. HIF-1a exhibits one bipartite (aa 17-33) and one monopartite (aa 718-721) NLS. Most proteins are translocated into the cell nucleus via the classical import pathway, mediated by importin alpha (and beta. So far, six human(alpha importins have been described. Here we show the specific binding of Hypoxia-Inducible-Factors to different importin isoforms and characterize the molecular mechanism of these protein-protein interactions.

G3–012P
The structure of calmodulin's complex with KAR-2: A novel mode of binding explains the drug's unique pharmacology

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KAR-2 (3'-(β-chloroethyl)-2',4'-dioxo-3,5'-spiro-oxazolidino-4-deacetoxy-vinblastine) is a potent antimicrotubular agent that

arrests mitosis in cancer cells without significant toxic side effect. In this study we demonstrate that in addition to targeting microtubules, KAR-2 also binds calmodulin, thereby countering the antagonistic effects of trifluoperazine (TFP). To determine the basis of both properties of KAR-2, the three-dimensional structure of its complex with Ca^{2+}-calmodulin has been characterized, both in solution using NMR and when crystallized using X-ray diffraction. Heterocorrelation (1H-15N-HSQC) spectra of 15N-labeled calmodulin indicate a global conformation change (closure) of the protein upon its binding to KAR-2. The crystal structure at 2.12 A-resolution reveals a more complete picture: KAR-2 binds to a novel structure created by amino-acid residues of both the N-and C-terminal domains of calmodulin. Though first detected by X-ray diffraction of the crystallized ternary complex, this conformational change is consistent with its solution structure as characterized by NMR spectroscopy. It is noteworthy that a similar tertiary complex forms when calmodulin binds KAR-2 as when it binds TFP, even though the two ligands contact (for the most part) different amino-acid residues. These observations explain the specificity of KAR-2 as an anti-microtubular agent: the drug interacts with a novel drug-binding domain on calmodulin. Consequently, KAR-2 does not prevent calmodulin from binding most of its physiological targets

G3–013P
Investigation of enolase and phosphoglycerate mutase interaction using molecular dynamics simulation

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Glycolytic enzymes are responsible for conversion of glucose to pyruvate. It is well known that glycolytic enzymes can form dynamic complexes and substrate channeling in such complexes may take place. CHARMM academic program has been used for energy minimization and leapfrog molecular dynamics to investigate further interaction between enolase and phosphoglycerate mutase (PGM) that has been shown experimentally in the *in vitro* conditions. To determine whether this interaction is "visible" with the help of computer modeling method, two different enolases have been chosen from available protein data banks. *Saccharomyces cerevisiae* PGM (1QHF) and enolase (2ONE) dimers have been tested for interaction. The results have been compared with results of interaction between *Trypanosoma Brucei* (TB) enolase monomer (1OEP) and the same yeast PGM. 3-phosphoglycerates of both subunits of PGM have been replaced with 2-phosphoglycerates (2PG). Enzymes were placed in a water box (with 10 Å separation), which was additionally extended for 30 Å in each X, Y and Z directions. Different orientations of enolase (30° step in each X, Y and Z direction) were used during simulation. For each orientation water box contained approximately 25 000 water molecules. During the simulation process, which took approximately 24 192 CPU h, the orientation of PGM was chosen to face its 2PGs to enolase to put them in a favorable position for possible channeling. Comparison of results showed that 66% of most active binding residues of TB enolase are identical to residues of yeast enolase. This is in a good agreement with *Trypanosoma* and yeast enolase residue sequence identity, which may suggest interaction specificity of some degree. Nevertheless no channeling has been recorded.

G3–014P
Synthesis and characterization of new alpha-conotoxin analogs in search of the effective and selective nicotinic acetylcholine receptor ligands

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α-Conotoxins (αCtx), short toxic peptides from poisonous sea snails of *Conus* genus, are widely used in current studies on the nicotinic acetylcholine receptors (nAChRs). Among the advantages of αCtx as nAChR antagonists are the simplicity of their synthesis and the distinct specificity to the different nAChR subtypes. Because of the importance of some nAChR subtypes in a number of neurodegenerative and mental disorders, design of more effective and selective compounds targeting with high affinity a particular receptor subtype is an urgent task. To examine the role of the charged groups in αCtx molecules in their activity and specificity, we synthesized a set of peptide analogs based on naturally occurring αCtx targeting both muscle- and neuronal-type nAChRs. The substitutions of uncharged or negatively charged amino acid residues for Lys or Arg in the Ñ-terminal part of α-conotoxins GI, MI, SI, SIA and PnIA were made. The synthesized analogs together with unmodified peptides were compared in their ability to interact with the native membrane-bound muscle-type nAChR from *Torpedo californica* ray electric organ as well as with the heterologously expressed acetylcholine binding proteins (AChBP) from *Lymnaea stagnalis* and *Aplysia californica* snails which resemble spatial arrangement and a number of pharmacological ligand-binding features of the extracellular domain of neuronal α7 nAChRs. It was shown that the incorporation of a positive charge in 12th position of the muscle-type αCtx amino acid sequences produces an increase in the affinity for the *Torpedo* nAChR. In the case of D12K-analog of αCtx SIA, the magnitude of this increase was two orders, rendering this compound the most effective antagonist of muscle-type nAChR among all tested peptides. A similar D14K-substitution in PnIA analog, acting on neuronal nAChR, enhanced more than ten times the affinity for *Lymnaea* but not for *Aplysia* AChBPs.

G3–015P
Interaction between enolase isozymes and cytoskeleton: a control mechanism of glycolytic energy utilization.

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Multiple interactions of glycolytic enzymes have been demonstrated. These interactions result in the local production of ATP at the appropriate subcellular sites [1]. Our studies with enolase, a glycolytic enzyme that exhibits tissue specific isoforms, have shown that isoenolases differ in their abilities to interact with other macromolecules. Therefore, they can be localized at different subcellular sites and respond to specific functional demands [2,3,4]. In adult mouse muscle, comparison of the expression of muscle-specific enolase and myosin heavy chain (MHC) isoforms demonstrated that levels of enolase immunoreactivity followed a

gradient that parallels the ATPase activity associated to MHC isoforms, being highest in fast glycolytic fibres IIB and barely detectable in slow oxidative fibres I [3]. Within the framework of a French Hungarian bilateral project, surface plasmon resonance (SPR) experiments were carried out to characterize quantitatively the specific interactions of enolase isoforms with tubulin. Our data showed that *in vitro* complex formations of tubulin and muscle- (or neuronal-) enolase are inhibited by the enolase substrate, 2-D-PGA. In order to reveal the physiological relevance of these interactions, immunolocalizations in various cell types are investigated by fluorescence microscopy. Our recent analyzes with confocal microscopy indicate that colocalization of enolase isoforms (alpha and beta) expressed in muscle cells with the microtubular network does occur at different steps of myoblasts differentiation. These studies are part of a global research program aimed to identify the control mechanisms involved in regulating energy utilization and production as a function of muscle physiological demands [5].

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G3–016P
TPPP/p25: a new natively unfolded protein and its connection with neurodegeneration

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We identified a brain-specific unstructured protein, TPPP/p25 that induced aberrant assemblies of microtubules *in vitro*. Here we present evidence that *in vivo* the target of this protein is the microtubular system. HeLa cells were transfected by a construct coding EGFP-TPPP/p25 fusion protein and the cells were investigated with different microscopic techniques. Time lapse and immunofluorescent experiments revealed that at certain level of the EGFP-TPPP/p25 the cell division was inhibited and the cell death was promoted. At extensive expression of TPPP/p25 two distinct structures was formed: TPPP/p25 was accumulated around centrosomes forming an aggresome-like structure protruding into the nucleus or microtubules were bundled into filamentous cage around the nucleus. These ultrastuctures were characterized at EM level as well. Then we search whether the TPPP/p25-induced aggresome formation is related to the formation of inclusions in neurodegeneration. The localization of TPPP/p25 was studied in post-mortem brain tissues from patients with confirmed neuropathology. Immunohistochemistry and confocal microscopy demonstrates that TPPP/p25 is enriched in filamentous alpha-synuclein bearing Lewy bodies of Parkinson's and diffuse Lewy body disease, as well as glial inclusions of multiple system atrophy. There is a correlation between TPPP/p25 and alpha-synuclein immunoreactivity in pathological samples. In contrast, TPPP/p25 is not associated with inclusions characteristic for tauopathies and corticobasal degeneration. We propose that a potential function of TPPP/p25 is the stabilization of physiological microtubular ultrastructures, however, its enhanced level may initiate directly or indirectly the formation of aberrant protein aggregates such as pathological inclusions.

G3–017P
The protein transport machinery of the mammalian ER membrane: *in vivo* effects of specific gene silencing

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The co-translational protein transport across the ER membrane is mediated by the protein-conducting channel Sec61p complex and associated proteins. The process is initiated when the signal sequence in a nascent polypeptide chain emerges form the ribosome. SRP (signal recognition particle) binds to the signal sequence and interacts with the ribosome at the same time. Thereupon the entire complex consisting of ribosome, the nascent chain and SRP binds to the ER membrane: SRP interacts with the SRP receptor and the ribosome is transferred to the Sec61p complex. Now the nascent chain can be translocated into the endoplasmic reticulum. In this study we repressed the expression of several proteins involved in this targeting process by RNAi. The interference with the intracellular protein transport caused specific effects concerning the cell viability as well as cell morphology.

G3–018P
Formation of ultrastructures induced by TPPP/p25 and its consequences

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Recently we identified TPPP/p25 as a brain-specific unstructured protein that induced aberrant microtubule assemblies and ultrastructure *in vitro*, and as a new marker of synucleopathies. The objective of this work was the identification of the interacting partners of TPPP/p25 and the elaboration of its intracellular function. NRK and HeLa cells were transfected with construct of EGFP-TPPP/p25 fusion, and the consequences of the protein's expression were monitored. We showed that TPPP/p25 colocalized with the microtubular network, the colocalization varied during mitosis, the reorganization of microtubular system is followed by TPPP/p25. The process of the cell cycle is not affected by low level of the protein, however, the accumulation of TPPP/p25 increased the number of cells in the phase of cytokinesis. The TPPP/p25 decorated microtubular system is resistant against depolymerization agents in *in vitro* and *in vivo* systems. In contrast, the association is dynamic demonstrated by FRAP experiments. High level of TPPP/p25 induces morphological and intracellular alteration in HeLa cells. Morphology of the cell becomes round, the microtubular network is disorganized, the protein accumulates at the MTOC and this aggregation -''aggresome''- protrudes nucleus. EM images revealed that TPPP/p25 is accumulated at the MTOC, as a centriole can be visualized in this region. We identified glyceraldehyde-3-phosphate dehydogenase within the ''aggresome'' as well. The formation of the protein aggregate affects the structure of the centrosome, as indicated by the separation of the two centrioles up to a few microns. In conclusion, our data suggest that the potential function of TPPP/p25 at physiological conditions could be the stabilization of microtubular system which might has special significance in neuronal cells.

G3–019P
Normal mode dynamics of the antigen HEL-antibody D1.3 complex in relation to the significance of the Fab constant domains in the complex formation

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Normal modes have been calculated from three dimensional structures of Hen Egg Lysozyme (HEL) with the fragment of antigen binding (Fab) of D1.3, one of the monoclonal antibodies. The Ca atomic fluctuations of HEL-Fab can be thought related to the protein-protein interactions; it is consistent with HEL-HyHEL-10 case in our previous paper (*Protein Sci* 2003;**12**: 2125–2131), in which it is reported that the constant domains of Fab are important in the complex formation between HEL and HyHEL-10. In order to elucidate the significance of the Fab constant domains, the motions of isolated light (L) and heavy (H) chains of Fab were analyzed from a normal mode analysis point of view. The dynamics in Fab complex of HEL-D1.3 shows almost same fluctuations with Fab consisted of variable domains (Fv) and constant CLCH1 domains. However, isolated each chain of L and H shows completely different atomic fluctuations. Atomic vibration motions of the single L and H chains are bigger than those of Fab complex and Fab. Since the some increment of fluctuations are additional to the atomic motions as a whole, it is considered that a kind of domain motions could be produced between L and H chains. Consequently, it is insisted that the decrease of the domain motions of the L and H chains of Fab is the origin of the stronger complex formation between the antigen and antibody (Fab) than between antigen and antibody (Fv) without the constant domains.

G3–020P
Structure and heteroassociations of the TPPP/p25, a new brain-specific protein

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Recently we identified a brain-specific protein, denoted TPPP/p25 that induced aberrant microtubule assemblies *in vitro*, colocalized with the microtubular network *in vivo*, and was enriched in human brain inclusions characteristics to synucleinopathies. Here we show our structural studies of this new protein (isolated from bovine brain or expressed in *E.coli*) by means of various spectroscopic methods. The ^{1}H-NMR spectrum showed resonance lines broader as expected for a global protein with same molecular mass. The lack of signal dispersion suggests that TPPP/p25 is either unfolded or may have very little stable structure. These results are in accordance with the data of CD measurements, which showed 4% α-helix content. Vibration CD and IR spectra revealed the unfolded structure of TPPP/p25 with some β-sheet. In the solvent trifluoroethanol, which mimics the hydrophobic environment, the α-helix content of TPPP/p25 was increased to 35%. We identified two targets of TPPP/p25, tubulin and calmodulin (CaM). Direct interaction between TPPP/p25 and tubulin was revealed by CD measurements, the formation of positive difference spectrum indicated the enhancement of alpha helix content within the protein complex. The comparative titration data showed that the isolated bovine protein bound to tubulin with higher affinity than that of human recombinant protein. Our preliminary data suggest that the phosphorylation may play role in the distinct heteroassociations. The interaction between TPPP/p25 with CaM was studied by fluorescent spectroscopy in the presence of ANSA, a dye, which is specific for hydrophobic substructures in proteins. The complex formations of TPPP/p25 with tubulin or calmodulin were quantitatively characterized by surface plasmon resonance measurements, the affinity constants elaborated from the sensograms are Kd: 0.23 μM for tubulin and Kd: 0.05 μM for CaM.

G4–Protein–Protein Interactions in Blood Coagulation

G4-001
Interactions between the fibrinolytic and matrix metalloproteinase systems and atherothrombosis

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Remodeling of extracellular matrix (ECM) by the fibrinolytic and/or metalloproteinase systems plays a role in the pathogenesis of cardiovascular diseases such as thrombosis, restenosis and atherosclerosis. Studies in transgenic mice with deficiency of main components of both proteolytic systems revealed a role in neointima formation after vascular injury. Indeed, deficiency of urokinase-type plasminogen activator (u-PA) or plasminogen reduces arterial restenosis, whereas deficiency of tissue inhibitor of MMPs type-1 (TIMP-1) enhanced it. In atherosclerotic lesions, plasmin generated via macrophage-secreted u-PA can activate several proMMP's. Mice with combined deficiency of apolipoprotein E (ApoE) and stromelysin-1 (MMP-3), kept on cholesterol-rich diet, showed enhanced atherosclerotic plaque burden but reduced aneurysm formation, whereas combined deficiency of ApoE and TIMP-1 resulted in reduced plaque size but more severe aneu-

rysms. Thus, active MMPs may contribute to destabilization of atherosclerotic plaques (by degrading ECM components), but may also promote aneurysm formation (by degradation of elastica lamina). Both proteolytic systems may represent therapeutic targets for treatment of atherothrombosis.

G4-002
Clot busting drugs studied using fluorescent protein fusions.

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Fluorescent protein fusions offer advantages over chemically labeled proteins, for example in binding studies which are needed for a full understanding of the regulation of fibrinolysis and "clot busting drugs". We have constructed and expressed a C-terminal fusion of human tissue plasminogen activator (tPA) with a commercial variant of the jellyfish Green Fluorescent Protein, EGFP (tPA-EGFP), using a baculovirus expression system with sf9 insect cells. tPA-EGFP was purified, and SDS PAGE confirmed the predicted molecular weight of 87 kDa. The activity of tPA-GFP was investigated in assays with an amidolytic substrate (S2288), and with plasminogen substrate in a fibrin-based assay. K_m for tPA-EGFP on S2288 was 1.45 μM and not significantly different from wt tPA expressed in insect cells (itPA) or International Standard tPA, (K_m around 1.7 μM). In a fibrin-based plasminogen activation assay, the K_m values for lys-plasminogen were 46, 54, and 86 nM for itPA, IS tPA, and tPA-EGFP, respectively. These results show that substrate binding is only marginally compromised by the fusion of the GFP to the tPA protease domain. Changes in fluorescence intensity of up to 20% were observed with the addition of templates that bind to tPA (fibrinogen or fibrin analogs) and plasminogen substrate. We have also constructed, and are expressing, Yellow Fluorescent tPA and Cyan Fluorescent plasminogen for Fluorescence Resonance Energy Transfer (FRET) studies for quantitative investigation of the assembly of tPA and Plasminogen on fibrin templates to aid our understanding of fibrinolysis and the treatment of acute myocardial infarction.

G4-003
Regulation of thrombin

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Following initiation of coagulation as part of the haemostatic response to injury, thrombin is generated from its inactive precursor prothrombin by factor Xa as part of the prothrombinase complex. Thrombin then has multiple roles. The way in which thrombin interacts with its many substrates has been carefully scrutinized in the past decades but until recently there has been little consideration of how its many functions are coordinated or directed. Any understanding of how it is directed requires knowledge of its structure, how it interacts with its substrates and the role of any cofactors for its interaction with substrates. Recently, many of the interactions of thrombin have been clarified by crystal structure and site directed mutagenesis analyses. These have revealed common residues used for recognition of some substrates and overlapping surface exosites used for recognition by cofactors. As many of its downstream reactions are cofactor driven, competition between cofactors for exosites must be a dominant mechanism that determines the fate of thrombin. This review will draw together much recent work that has helped clarify structure function relationships of thrombin. It will then attempt to provide a cogent proposal to explain how thrombin activity is directed during the haemostatic response.

G4-004
The anticoagulant protein C pathway

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The protein C pathway controls coagulation by regulating factors VIIIa and Va, cofactors in the activation of factor X and prothrombin, respectively. The pathway comprises membrane-bound and circulating proteins that assemble into multi-molecular complexes on the surface of different cells. The key component protein C circulates as zymogen to an anticoagulant serine protease. It is activated on endothelial cells by thrombin bound to thrombomodulin. Another membrane protein, the endothelial protein C receptor (EPCR), stimulates the activation. Activated protein C (APC) inhibits coagulation by cleaving peptide bonds in each of FVIIIa and FVa. This occurs on the surface of negatively charged phospholipid membranes and the reactions are supported by protein S. FVIIIa degradation by APC requires not only protein S but also the intact FV. Thus, FV has both pro and anticoagulant properties. The anticoagulant effects of protein C are physiologically important and genetic defects affecting the pathway are common risk factors of venous thrombosis. APC also has anti-inflammatory and anti-apoptotic functions. The physiologic importance and the mode of action of these activities are not well understood. They are reported to be dependent on the binding of APC to EPCR and the subsequent proteolytic cleavage of PAR-1. The proteins of the protein C pathway contain multiple domains and the three-dimensional structure of several of the proteins is known. The molecular recognition of the protein C system is beginning to be unraveled and we start to get insights into this fascinating and intricate molecular scenario at the atomic level.

G4-005
Fibrinolysis in phospholipid environment: modulation through release of fatty acids

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Thrombolysis is conventionally regarded as dissolution of the fibrin matrix of thrombi by plasmin, but thrombi contain various components (proteins, phospholipids), which profoundly affect the process of solubilization. We have recently shown that phospholipids present in arterial thrombi at millimolar concentration significantly retard tissue-type plasminogen activator (tPA) induced fibrinolysis. Our present measurements evidence that in turbidimetric fibrinolytic assay 1.4 mM phospholipid prolongs the tPA-induced lysis time from 40 to 60 min, but partial destruction of the phospholipid barrier with phospholipase A2 relieves this inhibition (lysis time 47 min). Complete hydrolysis of the ester bond at position two of the phospholipid, however, generates millimolar concentration of fatty acids, which have independent effects on the fibrinolytic system. When tPA activity is measured on a small synthetic peptide substrate (Spectrozyme tPA), oleic acid (1.4 mM) increases the K_m of tPA from 0.12 to 0.63 mM, whereas stearic acid (1.4 mM) decreases the Vmax 4-fold. Plasmin activity on a chromogenic substrate (Spectrozyme PL) is completely inhibited by these concentrations of oleic acid, but fibrin partially prevents this effect (dissolution of fibrin clots is slowed down to a lesser degree than expected from the inhibition of the amidolytic activity). Interestingly, using an assay focusing on the

plasminogen activation step within fibrin plasmin generation by tPA is even accelerated by oleic acid on the surface of the clot in certain stages of the process. Thus, the overall course of thrombolysis *in vivo* is probably largely dependent on the balance of phospholipids and phospholipases released from the cellular constituents of thrombi.

G4-006
Factor VIIIa regulates substrate delivery to intrinsic tenase

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Coagulation factor X (fX) activation by activated factors IX (fIXa) and VIII (fVIIIa) requires assembly of the enzyme-cofactor-substrate fIXa-fVIIIa-fX complex on phospholipid membrane. To study the mechanism of the fX-activating complex assembly, we focused on formation of the intermediate membrane-bound binary complexes of fIXa, fVIIIa, and fX. Flow cytometry studies of coordinate binding of fluorescein-labeled factors to 0.8 μM phospholipid vesicles (25/75 phosphatidylserine/phosphatidylcholine) demonstrated that fVIII, fIXa, and fX bind to 32 700 ± 5000, 20 000 ± 4500, and 30 500 ± 1300 binding sites per vesicle with apparent K_d of 76 ± 23, 1510 ± 430, and 223 ± 79 nM respectively. FVIII at 10 nM induced appearance of additional high-affinity sites for fIXa (1810 ± 370, 20 ± 5 nM) and fX (12 630 ± 690, 14 ± 4 nM), while fX at 100 nM induced appearance of high-affinity sites for fIXa (541 ± 67, 23 ± 5 nM). FVIII effect did not require activation by thrombin. Parallel titrations of fIXa, fX and fVIII (or fVIIIa) demonstrated correlation between fVIIIa binding and appearance of binding sites for fIXa and fX, indicating formation of 1:1 complexes between these factors. Kinetic binding studies have shown that the presence of fVIII increased not only affinity, but also the rate of fX association with lipids. The apparent Michaelis constant of fX activation by fIXa was a linear function of fVIIIa concentration, with the slope of 1.00 ± 0.12 and intrinsic K_m of 8.0 ± 1.5 nM. There was correlation between FX activation rate and the fVIIIa-fX complex formation. Studies of fX activation at varied concentrations of fX, fVIIIa, phospholipid and phosphatidylserine content suggested that at high fVIIIa concentration (12 nM) substrate was delivered to the fVIIIa-fX complex without prior association with lipids, i.e. following the "free substrate" model. Summarizing, results of this study indicate that: (i) fVIII and fVIIIa provide high-affinity binding sites for fX on phospholipids; (ii) formation of the fVIIIa-fX complex on the membrane regulates fX activation; (iii) the mechanism of this regulation is likely to be delivery of fX to the membrane-bound enzymatic complex.

G4-007P
Integrin alphallbbeta3 activation mechanism mediated by adrenergic stimulation in a heterologous model

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A requisite for platelet aggregation is the binding of soluble fibrinogen (Fg) to cell surface receptors. Fg receptor (integrin αIIbβ3) is normally maintained in a resting state and is activated by "inside-out" signals that are transmitted through the tails of the αIIbβ3 complex to its ectodomain. Under physiologic conditions, signals are generated by the stimulation of G protein coupled receptors such as purinergic and adrenergic, and the actual nature of the signals generated by these agonists remains unclear. The present work aimed at analyzing whether the stimulation of human recombinant α2A-adrenergic receptors (α2A-AR) could lead to a full activation of reconstituted Fg receptors. CHO cells coexpressing α2A-AR and human Fg receptors aggregate in response to epinephrine in a calcium, αIIbβ3, and soluble Fg-dependent manner that is prevented by α2A-AR antagonists or αIIbβ3 blockade. The α2A-induced activation of αIIbβ3 in CHO-αIIbβ3-α2A-AR cells increased the rate of adhesion and spreading of cells onto Fg coated plates. PKC inhibitors prevented this effect on cellular adhesion but had no effect on cellular aggregation. The adrenergic-induced cellular aggregation was not related to the decrease in intracellular levels of cAMP caused by α2A-AR stimulation, but was associated with the stimulation of a calcium/calmodulin-dependent signaling pathway. The α-adrenergic activation was associated with phosphorylation of a protein of ~100 kDa and proteins of the MAPK family. This model has allowed to differentiate intracellular signaling pathways distinctly associated with α2A-AR-induced stimulation of either aggregation or adhesion in a reconstituted αIIbβ3 system.

G4-008P
Metronidazole effect on lowering blood serum lipid and proteins

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Introduction: Hyperlipidemia is one of the most important cause of atherosclerosis. Regarding the high prevalence of this disease in the world especially in Iran, finding suitable and effective treatment which can both control the level of blood lipids and solution should be the major aim. Metronindazole is the only drug that is used as an antibacterial and antiprotozoal agent. Researchers have found that oral dose of 750 mg/day has a suitable absorption, is widely spread in the tissues and reaches to a serum level of 4–6 g/ml. Despite its suitable efficiency, a few clinical trials have been conducted in this regard. This study was designed as such to examine effect of Metronidazole on lowering serum lipids.
Methods: The present research was performed as a clinical trial without control, on 50 patients. The tests of LFT, BUN creatinin, SGOT and SGPT were done on these subjects. Metronidazole was given with a daily dose of 750 mg for a week. 20 of these patients who had less complaint continued their drug consumption for another seven days. Lipid parameters such as total lipid, total cholesterol, and triglycerides were taken under investigation in both groups.
Results: Measuring the serum level of lipids indicated that the mean total serum lipid and total cholesterol decreased significantly compared to their level before using the drug ($P < 0.05$). The findings also showed a similar decrease in serum TG level ($P < 0.05$). In the patient who continues the drug consumption in the second week, the serum level of lipids under study decreased significantly compared to their level in the first week ($P < 0.01$).
Discussion: Drug tolerance indicates appropriate action and metabolism in this regard. This can be a prognosis for the replacement of this drug with other in the treatment of hyperlipidemia. The significant increase of BUN shows the small changes in liver function as if the drug should be used for a long time in order to treat the hyperlipidemia, requires a more accurate investigation. Altering the daily dose of the drug should also be considered.

G4-009P
Purine bases in advanced carotid artery plaque

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Siems *et al.* (1994) [1] demonstrated a rapid decrease in ATP during hypoxia/reoxygenation. The degree of ATP loss was correlated inversely with rate of ATP restoration during posthypoxic reoxygenation. A drastic increase in catabolic pathways was observed during anoxia and normalization of flux rates during reoxygenation. Activities of HGPRT and APRTase were much lower, whereas those of 5'-nucleotidase and AMP deaminase were dramatically enhanced. This involved accumulation of free bases and their products, hypoxanthine (Hx) and xanthine (X). Increased posthypoxic free radical formation leads to accumulation of hypoxanthine and major xanthine oxidase activity [2]. Arterial wall endothelial cells, which are continuously exposed to changing oxygen pressure and high free radical formation, presumably modify purine nucleotide content, and this would be reflected by plaque on arterial walls. We therefore measured uric acid (UA), Hx, X, allantoin (ALL), free radical (FR) and sulphydryl (SH) group content of plaque and plasma of 50 patients with carotid plaque. The results, expressed as mean and standard deviation, were: in plaque, FR = 503 ± 348 μmol_{H2O2}/g; SH = 1.59 ± 0.39 μmol/g; UA = 0.061 ± 0.041 μmol/g; Hx = 0.263 ± 0.112 μmol/g; X = 0.077 ± 0.053 μmol/g; ALL = 0.86 ± 0.15 μmol/g; in plasma, FR = 4.67 ± 1.15 μmol_{H2O2}/ml; SH = 0.326 ± 0.083 μmol/ml; UA = 0.33 ± 0.10 μmol/ml; Hx = 0.0013 ± 0.0009 μmol/ml; X = 0.0058 ± 0.0038 μmol/ml; ALL = n.d. FR, Hx, X were much higher in plaque than plasma. Moreover, ALL is evident, due to chemical oxidation of UA, because humans do not have the enzyme uricase. On the other hand, SH and UA, oxidant defenses, were higher in plasma. We conclude that oxidative stress is evident in advanced plaque and that besides the well-known components of plaque, there are other compounds, which could be involved in the pathogenesis of the disease and could be useful in preventive chemotherapy.

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G4-010P
Molecular structure and antithrombotic function of disintegrin

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Glycoprotein IIbIIIa (GPIIbIIIa) is a platelet integrin receptor that is considered to be the final common pathway of platelet aggregation in human blood vessel. When platelets are activated, the receptor undergoes conformational change and binds the RGD (Arg-Gly-Asp) sequence of various protein ligands. Platelet aggregation takes place by cross-linking the cells through GPIIbIIIa binding to fibrinogen, which is the major RGD-bearing protein in blood stream. Therefore, a powerful GPIIbIIIa receptor antagonist is expected to effectively prevent thrombus formation. Previous reports in this laboratory have demonstrated molecular cloning, protein purification and characterization of a snake venom-derived disintegrin that strongly inhibits platelet aggregation. The NMR solution structure of the disintegrin revealed that a conserved RGD motif with an unusual finger shape is distal from the rigid core of the C-terminal domain. Although the RGD motif does not interact with the hydrophobic core of the protein, the 3D structure is stabilized by a network of contacts through a small antiparallel beta-sheet. The disintegrin inhibits GPIIbIIIa binding to immobilized fibrinogen with an IC50 of 2.2 nM and ADP-induced human platelet aggregation with an IC50 of 131 nM, respectively. Acute and sub-acute toxic effects of the disintegrin were examined in mice. The half-life of the protein in the blood stream was also determined in animal model. Taken together with its molecular structure of the antithrombotic disintegrin, *in vitro* and *in vivo* experimental data will be demonstrated in view of the development of a platelet aggregation inhibitor.

G4-011P
New polymerization sites functioning at two stages of fibrin self-assembly.

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Fibrin (f) polymerization is carried out by two pairs of complementary polymerization sites "A"-"a" and "B"-"b". However there is a lot of evidence that additional sites in f molecule play role in this polymerization. Using our monoclonal antibody (mAb) NI to D-dimer we found previously unknown polymerization site (named "c") participating in f protofibril formation. This site is situated in NH2-terminal part of D-domain gamma-chain. We suggest that complementary site ("C") in E-domain is situated in Bbeta15–53 fragment of f molecule, namely at Bbeta41–45 and/or Bbeta27–31. Our mAb NII and its Fab retard to 50% the rate of protofibril formation at equimolar ratio to f. Epitope for mAb NII proved to be localized at f fragment Bbeta15–53. Another mAb NIII with an epitope encompassing peptide bond Bbeta Arg14–Gly15 inhibited to 60% lateral association of f desAA protofibrils at equimolar ratio to f and its Fabs retarded to 100% this process at ratio to f = 2. Thus the unknown site of protofibril lateral association is situated in f desAA fragment Bbeta14–25, which functions before splitting off fibrinopeptide B. When fibrinopeptide B is split off and polymerization site "B" is exposed the latter binds to the site "b". We suggest that the part of the site Bbeta14–25, may be Bbeta20–25, which is situated at the right side of peptide bond 14–15 keeps binding to betaC-domain of another f molecule. Our mAb NIV to epitope in Bbeta15–53 inhibited to 100% protofibril lateral association of f desAA and of f desAABB. We suggest that f fragment Bbeta14–53 includes the unknown sites, one of which participates in f protofibril formation stage and another one in protofibril lateral association stage.

G4-012P
Dysfunctional regulation of vesicular trafficking and membrane homeostasis in human platelets

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Secretory lysosomes are cell-specific modifications of the post-Golgi endomembrane system that undergo regulated secretion in response to external stimuli. The d-granules can be regarded as the secretory lysosomes of platelets. Undisturbed vesicular transport and fusion of the granules with the plasma membrane are prerequi-

sites for a sufficient release reaction and platelet aggregation. In addition, the coagulation amplification on the platelet outer membrane depends on the supply of phospholipid compounds that facilitate the tenase and prothrombinase complex formation. Different pathways are involved, e.g. cargo proteins as the adapter proteins (AP) or the biogenesis of lysosomal-related organelle complexes (BLOC) are necessary for a fully functional vesicular trafficking. The underlying mechanism of dysfunctional AP or BLOC are mutations in the HPS genes. The transmembraneous SNARE proteins are essential components of the vesicle fusion machinery. Interacting vesicle-associated membrane proteins (VAMP), especially VAMP-3 and -8, are required for granule secretion as well. Furthermore, the phospholipid composition of the platelet membrane leaflets is maintained by scramblases, translocases etc. but also by ABCA family transporters. A defective vesicular trafficking and a disturbed membrane homeostasis represent novel mechanisms for bleeding disorders. It can be identified by a diminished binding of annexin V, factor Va and VIIIa to the activated platelet. The endogenous thrombin potential of platelet-rich plasma is affected, i.e. limited phosphatidylserine exposure reduces both onset and rate of thrombin generation but not the total amount of thrombin. The mass-spectrometric evaluation of the platelet membrane reveals an altered phospholipid pattern.

G4-013P
Thrombolytic activity of annexin XI and V fusion protein with staphylokinase

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Thrombolytic therapy has been a major advance in the treatment of acute myocardial infarction over the last decade. However, available thrombolytic agents have certain limitations. The thrombus may be resistant to lysis in some patients or may reform after initial lysis; and intracerebral bleeding is particularly a serious side effect. The reformed secondary clots are usually platelet-rich and show strong resistance to lysis mediated by t-PA, the most commonly used thrombolytic agent. To address these shortcomings, we report the engineering of staphylokinase-annexins fusion protein. Staphylokinase is a highly fibrin selective thrombolytic agent. Annexins, a family of human proteins, bind with very high affinity to the phosphatidyl serine molecules exposed on activated platelets in the presence of calcium. The genes of fusion proteins were constructed on pET24 a(+) with staphylokinase placing at the N-terminal. The proteins were expressed in and purified from BL21 using his-tag chelating column. The purified fusion proteins were shown capable of lysing fibrin clot and good activity as a plasminogen activator. It was found that annexins alone showed plasminogen activator activity, though weaker than staphylokinase. The plasminogen activator activity of fusion proteins was not synergistically increased. Binding to phosphatidyl serine on phospholipid vesicle was demonstrated. Tests of lysis of blood clot with high platelet content are undergoing.

G4-014P
Co-localization of caveolin proteins and AQP-1 may mediate in vasopressin induced hemostasis

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The synthetic analogue of vasopressin, 1-Deamino-8D-AVP (DDAVP) is predominantly a V2 receptor agonist. DDAVP has been widely used in the treatment of von Willebrand's Disease, hemophilia A, platelet disorders and Uremic bleeding. DDAVP and Arginine vasopressin (AVP) stimulate the endothelial V2 receptor and release von Willebrand factor and coagulation factor VIII. However the cellular mechanisms leading to the enhanced release of clotting factor VIII and von willebrand factor remains to be understood. This study was conducted to evaluate the role of caveolin proteins and aquaporin-1 water channels (AQP-1) in the hemostatic actions of vasopressin. Groups of mice ($n = 6$) were treated with AQP-1 blocker, tetraethylammonium (30 mg/kg i.p) and caveolin modulator, cholera toxin (200 μg/kg sc) and evaluated for hemostasis via noting the bleeding time. These animals later received an injection of AVP and DDAVP (5 μg/kg i.p). The results of our study suggest that pretreatment with caveolin modulator and AQP-1 blocker enhanced the hemostasis observed after treatment with vasopressin analogues. These results suggest that AQP-1 water channel and caveolin proteins are possibly colocalized and contribute to modulate the hemostatic mechanisms of vasopressin. These results also suggest the involvement of AQP-1 and caveolin in the cellular mechanisms for vasopressin induced hemostasis.

G4-015P
Is there a role for the generation of two-chain tissue plasminogen activator from its active proenzyme in blood clot lysis?

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Tissue plasminogen activator (tPA) is a serine protease of the chymotrypsin family, however unlike other family members the single-chain "proenzyme" (sctPA) is active, rather than a zymogen. tPA activates the zymogen plasminogen to plasmin, which degrades fibrin in blood clots and plasmin also converts sctPA to the two-chain form (tctPA). However, there are contradictory reports on the relative activities of sc- and tctPA. As part of our efforts to model the kinetics of fibrin-bound plasminogen, we have expressed recombinant tPA using a baculovirus system with sf9 insect cells (itPA, expressed and purified in a sc form), and a mutant non-cleavable form (sctPA:R275E). tctPA was generated from plasmin-treated itPA. In line with previous reports, tctPA was 3-fold more active on an amidolytic substrate than itPA and sctPA:R275E. However, when activating plasminogen, sctPA:R275E was 3–6-fold (at low and high plasminogen concentration ranges, respectively) less active than itPA or tctPA, indicating that itPA is rapidly converted to tctPA when plasmin is generated. The addition of fibrinogen to this system stimulated plasminogen activation rates by all tPA forms (up to 20-fold for sctPA:R275E) so that all tPA variants had the same activity. Similarly, no differences in tPAs were seen in the presence of fibrin clots under a variety of conditions. Since all tPAs have equivalent activity in fibrin, proteolytic generation of tctPA, may be part of a deactivation pathway in circulation since PAI-1 (a specific serpin inhibitor) is reported to be more active against tctPA. Thus, sc- to tctPA conversion does not stimulate fibrinolysis but could be important in other systems such as brain function and ovulation.

G4-016P
The regulation of Von Willebrand Factor A1 domain GPIb-IX-V interaction

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Von Willebrand factor (VWF) is an important player of haemostasis, as it supports platelet adhesion to subendothelial structures.

Although it is present in circulation, it does not spontaneously bind to its platelet receptor, GPIb-IX-V. High shear stress and/or immobilization enables this interaction; probably by inducing a conformational change. *In vitro*, a similar effect is achieved with the previously described monoclonal antibody 1C1E7. GPIb-IX-V binding is mediated by the A1 domain, whereas, 1C1E7 binds in the N-terminal (D'D3) region. Our hypothesis is that this region is important in the regulation of GPIb-IX-V binding, possibly by shielding the A1 domain. Based on the finding that 1C1E7 does not bind to porcine VWF, we made human/porcine VWF chimeras and found that K967 is essential to maintain its epitope. Next, ristocetin-induced platelet agglutinations were performed. The N-terminal chimeric VWF or VWF with the D'D3 region deleted (ΔD'D3) had an increased sensitivity in the ristocetin-induced agglutination, suggesting that this region has an inhibitory role. Moreover, ΔD'D3 requires less ristocetin than D'A3 VWF to bind to a recombinant GPIbα fragment. Next, the conformation of the A1 and the N-terminal domains was inspected. Cross-competition between antibodies binding to these domains indicates their proximity. Inhibition of ΔD'D3 by D'D3 VWF in ristocetin induced platelet agglutination is a sign of direct interaction of the A1 and the N-terminal domains. We determined the AA sequence of 1C1E7 and modeled its structure. Based on sequence similarity and structure, we could deduce that 1C1E7 may mimic the A1 domain.With the present experiments, we here provide strong evidence for our hypothesis that in non-sheared VWF the A1 domain is shielded by the D'D3 domain.