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Conference Committee

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Abstracts

LECTURES - MARCH 13

POLYMER CARRIERS FOR TARGETED DRUG DELIVERY AND CONTROLLED DRUG RELEASE

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Nondegradable *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers or biodegradable poly(ethylene glycol) (PEG) multiblock copolymers conjugated with antibodies were designed as water-soluble carriers of anticancer drugs facilitating site-specific therapy. High-molecular-weight polymer carriers were used for passive targeting to solid tumors while conjugates with specific polyclonal or monoclonal antibodies were designed for specific delivery of anti-cancer drug doxorubicin to tumor cells or model tumors inoculated in mice. Doxorubicin conjugates targeted with B1 monoclonal antibody (mAb) were shown to possess strictly tumor-specific binding capacity to target BCL1 cells *in vitro* and superior *in vivo* activity to free doxorubicin or non-targeted polymer drug in the treatment of established BCL1 leukemia in mice. Likewise, the use of conjugates targeted with anti EL4 mAb resulted in large amount of long-term survivors after treatment of mice bearing mouse EL4 lymphoma. Preliminary evaluation of doxorubicin conjugate in human demonstrated that the polymer drugs rank among the most promising candidates for successful application in human cancer chemotherapy.

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LECTURES - MARCH 14

INTERACTION OF RHOA GTPASE WITH ITS EFFECTOR P160ROCK

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The GTP-binding proteins (or Rho-GTPases) of the Rho-family regulate a variety of cellular processes in all eukaryotic cells, ranging from cytoskeletal reorganization and cell motility to gene transcription in response to external stimuli [1]. To date, 19 different mammalian Rho-GTPases have been identified from which Cdc42, Rac1 and RhoA are the most extensively characterised members of the Rho-family. The function of Rho-GTPases depends on the guanine nucleotide-bound state. As molecular switches Rho-GTPases cycle between an inactive GDP-bound state and an active GTP-bound state, which is controlled by numerous cellular proteins. Active form of Rho-GTPases interact with their downstream targets, so-called effector proteins, that are responsible for the diverse biological effects of Rho-GTPases [2]. One of the best studied Rho-Effectors, the Ser/Thr kinase p160Rock, plays a key role in actin-myosin filament assembly by activation of signalling molecules involved in various biological processes [3].

We will present the structure of the complex of RhoA GTPase with the Rho-binding domain of p160Rock. We found that the switching regions of RhoA molecule interacts with C-terminal part of parallel coiled-coil formed by Rho-binding domains. Such an arrangement of the complex will be discussed with respect to the general switching mechanism of GTPases and their interaction with downstream effectors.

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STRUCTURAL BIOLOGY APPROACH TO STUDY THE SPORULATION PROCESS

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Bacillus subtilis is a model organism for the study of one of the simplest cell differentiation process, called sporulation. A rich amount of genetic, biochemical and molecular biology data have been obtained during the study of this process. Recently, this study has advanced to the solving the tertiary structure of key protein regulators. This contribution focuses on the progress in protein crystallography oriented toward the understanding of sporulation mechanisms, that have been achieved in the last couple of years.

The phosphorelay is the main regulatory network in the initation of sporulation. Recently, the tertiary structures some of the components of this network were solved. The structures of response regulators Spo0F [5], Spo0A [2,3,4] and phosphotransferase Spo0B [7], together with biochemical and mutational data provide an important framework for further understanding of their biological function at the molecular level.

Structural data were also obtained from some proteins involved in activation of the first cell-type specific factor - ^{F} (structures of anti-anti- factor SpoIIAA and anti-factor SpoIIAB in the complex with ^{F}) [6,1].

The structures of only a small number from more than 100 sporulation specific proteins are known due to problems associated with the crystallization of these proteins. Structures for many of the interesting candidates remain a challenge. Among them are the membrane bound proteins and proteins with highly flexible domains.

Especially interesting for understanding of the mechanism of the transient gene expression asymmetry during sporulation would be the detailed study of sporulation septa formation by solving the crystal structure of key proteins involved in this cell division and chromosome translocation processes such as phosphatase SpoIIE, DNA translocase SpoIIIE or division protein DivIVA.

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STRUCTURAL BASIS OF PROTEIN METASTABILITY

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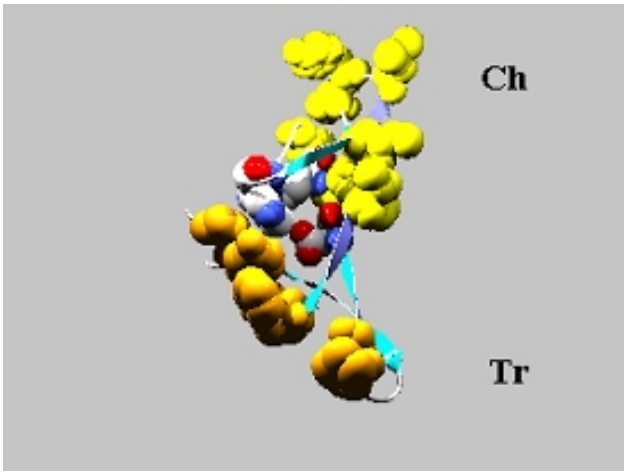
All information for the three dimensional structure of proteins and their functionality is encrypted within their amino acid sequences. The natural amino acid sequences of proteins have been perfected by evolution not only for their functional structure but also for a rapid and highly directional acquisition of their folded, fully functional state. An unambiguous distinction between these two possibilities is important for a clear-cut interpretation of consequences of amino acid replacements in protein engineering experiments.

The double-headed Bowman Birk serine protease inhibitor (BBI) built up from two triple stranded b-hairpin domains directed against trypsin and chymotrypsin was selected as a model protein. The double-headed arrangement of two independent subdomains facilitates the detection of long-range irregularities transmitted from the trypsin- into the chymotrypsin-inhibitory region used as a reference.

The exposed hydrophobic patches on the protein surface and a polar protein interior appear as structural peculiarities more reminiscent of the kind of structural features that have been postulated to occur in partially folded proteins rather than their folded state.

The autonomous refolding competence of the parent protein was abolished as s result of amino acid replacements, resulting in heterogeneous populations of conformers greatly differing in their activity. In contrast to the autonomous refolding competence of the parent protein, the variants require the presence of trypsin-Sepharose as a template with complementary structure in order to reach their fully active state. The fully active state of the variants attained by means of this method returns to the initial mixture of conformers upon subsequent incubation in the refolding buffer in a slow first order reaction. Therefore, the fully active state of the variants may be regarded as local energetic minima surrounded by high barriers of activa-



The exposed hydrophobic patch belonging to the trypsin-inhibitory region (Tr) is shown in ochre and that projecting out from the chymotrypsin-inhibitory region (Ch) is shown in yellow. Residues belonging to the buried polar interior are highlighted in CPK.

tion. The appearance of apparently metastable state supports a kinetic reaction control for the variants on the template although it cannot rule out a thermodynamic reaction control. In fact, the template facilitates folding not only kinetically, by reducing the high barrier of activation in solution but also thermodynamically by stabilising the fully active state by means of protein-protein-interactions. Protein metastability has also been documented for certain proteolytic enzymes after removal of their prosequences and for the native conformation of viral hemagglutinins. Presently, it is unknown whether the native conformation of soybean BBI corresponds to a global energy minimum or a metastable state on its conformational landscape. However, the inside-out situation in BBI and the conformational changes that are induced with reducing agents even in the absence of denaturants seem to be more in favour of the second possibility.

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SATURATION MUTAGENESIS OF L177 IN HALOALKANE DEHALOGENASE LINB

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Enzyme LinB is the haloalkane dehalogenase from bacterium *Sphingomonas paucimobilis* UT26. It is involved in a biochemical pathway for degradation -hexachlorocyclohexane. LinB catalyses hydrolytic dehalogenation of broad range of halogenated aliphatic compounds [1]. The amino acid in position 177 was identified as a very important determinant of catalytic properties of LinB by structural analysis [2] and by comparison of its protein sequence with other family members: (i) L177 is positioned in the mouth of the entrance tunnel leading the enzyme active site and is pointing directly to the tunnel and (ii) L177 is the most variable residue of the active site pocket among different haloalkane dehalogenases. L177 of the wild type enzyme was therefore replaced by every other amino acid and then the effect of mutations on enzyme activity was studied. Construction of the protein variants was conducted in two successive rounds. In the first round, L177 was replaced by A, C, G, F, K, T and W, respectively. The specific activities of the first set of mutants were statistically analysed but results from this analysis were not statistically significant. For that reason the first set of mutants was complemented with the second set of mutants comprising replacement of L177 by D, E, H, I, M, N, P, Q, R, S, V and Y, respectively.

All seven protein variants of the first set could be overexpressed in *Escherichia coli* and showed activity with at least some of the substrates used for characterization. In the second set, two out of twelve protein variants (L177E and L177N) could not be overexpressed in *E. coli*, while other two variants (L177P and L177I) did not show activity with any of the substrates. Circular dichroism spectra were recorded for all proteins purified in the second set and two inactive mutants showed spectra different from wild-type LinB and other mutants (Fig. 1), suggesting a decrease in the number of amino acids in -helical conformation and protein unfolding.

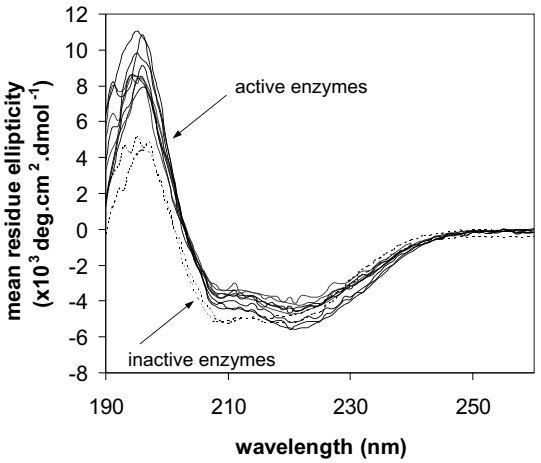

Fig. 1. Far-UV circular dichroism spectra of the wild-type haloalkane dehalogenase LinB, L177D, L177H, L177M, L177Q, L177R, L177S, L177V, L177Y mutants (solid lines) and L177P, L177I mutants (dashes lines). The spectra were measured at room temperature with the protein concentration 0.3 mg/ml in 50 mM phosphate buffer (pH 7.5) using the JASCO J-810 spectropolarimeter.

Successfully purified enzymes from both sets were kinetically characterized using a gas chromatography. Compounds 1-chlorobutane and 1,2-dibromoethane were selected as the substrates for steady-state kinetic measurements because they often serve as the reference compounds for characterization of the haloalkane dehalogenases. Dehalogenation of 1-chlorobutane showed typical Michaelis-Menten dependence, while dehalogenation of 1,2-dibromoethane showed substrate inhibition. Furthermore,

the specific activities of all prepared enzymes were determined for twelve different substrates (1-chlorobutane, 1-chlorohexane, 1-bromobutane, 1-iodobutane, 1,2-dichloroethane, 1,2-dibromoethane, 1,3-diidopropane, 1,2-dichloropropane, 1,2,3-trichloropropane, chlorocyclohexane, bromocyclohexane and 3-chloro-2- methyl propene) and statistically analysed by Principal Component Analysis. The first and only important component explained 45.8% of the data variance. Catalytic activity of mutant protein correlated mainly with the size of amino acid introduced to the position 177.

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STUDY OF ENZYME CATALYSIS USING TRANSIENT KINETIC AND MICROCALORIMETRY TECHNIQUES

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Kinetic studies in enzymology deals with elucidation of enzymatic reaction pathway, identification of reaction intermediates and with specification of the steps that limit the rate of turnover. The kinetic analysis of an enzyme mechanism often begins by analysis of the steady-state kinetics. The steady-state kinetic parameters, K_m and k_{cat}, provide information sufficient to describe a minimal kinetic scheme. Conclusions that can be derived by steady-state analysis are considered preliminary. Because the steady-state kinetic parameters are complex functions of all the reactions occurring during enzymatic catalysis, individual reaction steps are buried within these terms and cannot be resolved. These limitations are overcome by examination of the reaction pathway by transient kinetic methods, where the enzyme is examined as a stoichiometric reactant, allowing individual steps in pathway to be established by direct measurement. Steady-state and transient-state kinetic studies complement each other in elucidation of enzymatic reaction pathway. Analysis in the steady-state should be a prelude to the proper design and interpretation for more detailed transient-state experiments [1].

Both steady-state and transient-state kinetic methods were applied to solve reaction pathway, to identify reaction intermediate and to specify the rate limiting step of catalytic action of haloalkane dehalogenase LinB from bacterial strain *Sphingomonas paucimobilis* UT26 [2]. Steady-state experiments involved direct monitoring of LinB activity by isothermal titration calorimetry and initial rate of product formation measurements by gas chromatography. Transient-state kinetics used stopped-flow fluorescence and rapid-quench-flow techniques. Additional steady-state inhibition experiments and transient-state binding experiments were employed to find out leaving ability of both products (a halide and an alcohol) during dehalogenation reaction.

The results showed that export of products as well as import of substrates into the active site of LinB are fast processes reaching rapid equilibrium. This fast exchange of the ligands between the active site and bulk solvent can be explained by wide opening of the entrance tunnel and large active site of LinB. In contrary, the release of the halide ion from narrow active site after the reaction was found to be slow rate limiting step for another haloalkane dehalogenase, enzyme DhlA from *Xanthobacter autotrophicus* GJ10 [3]. The actual cleavage of the carbon-halogen bond was found to be fast step in both enzymes.

The rate of cleavage of C-Br bound is faster than cleavage of C-Cl bound, which is in agreement with bromide being a better leaving group in biomolecular nucleophilic substitution than chloride. This observation correlates with the lower affinity of LinB for chloride compared to bromide. Further the results confirmed, that the reaction proceeds via a covalent alkyl-enzyme intermediate. Using bromocyclohexane, chlorocyclohexane and 1-chlorohexane as model substrates, hydrolysis of this intermediate was found to be the slowest step in the catalytic cycle of LinB. The alkyl-enzyme complex was highly accumulated due to the fast dehalogenation step following the slow hydrolyses of this intermediate. The study provides a basis for the analysis of kinetic steps in hydrolysis of environmentally important substrates by the action of LinB.

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LOCALISATION OF ACCUMULATED CHLOROPHYLL CATION IN REACTION CENTRE OF PHOTOSYSTEM II

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Photosystem II reaction centre absorbance and circular dichroism spectra were calculated using point-dipole approximation and compared with experimental data. Light induced difference spectra and their calculated counterparts revealed the location of accumulated chlorophyll cation at the position Chl1 - the accessory chlorophyll at D1 subunit.

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SPECTRAL TECHNIQUES FOR RAPID QUANTIFICATION OF PROTEIN STRUCTURE IN SOLUTION

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Development and improvement in techniques that can be used for characterization of proteins is becoming increasingly important in the rapidly expanding field of proteomics. NMR techniques can provide high-resolution structures of proteins in solution essentially equivalent in quality to those determined by crystallography. Unfortunately, these methods are still time consuming, require milligrams of protein and are limited to proteins of low molecular weight.

Optical spectra such as electronic [1-2] and vibrational [3] circular dichroism (ECD and VCD), Fourier transform infrared (FTIR) [4], as well as Raman [5] spectra have been extensively used to obtain estimates of the average fractional components of secondary structure in a protein. Moreover, these optical methods, which sample structure on an inherently rapid time scale, are particularly appropriate for studying protein-folding processes [6,7], since the intermediate species one wishes to study are often unstable. Such dynamic structures are poorly suited to more precise, but slower time scale, NMR structural techniques or to X-ray diffraction analyses of the crystal-stabilized distribution of structures. Optical spectra can rarely yield the structural detail of those techniques but remain vitally useful for qualitative monitoring of the structure, particularly for relative changes in a single or related protein.

For most applications, no single spectroscopic technique can provide all the information needed, and multitude of methods must usually be employed in order to meet all these needs. For instance, ECD results can often be difficult to interpret since aromatic amino acids can interfere. A reasonable strategy in this case is to confirm the CD results with one of the vibrational spectroscopies - Raman, FTIR or VCD - owing to spectral separation of structurally characteristic vibrational modes. Both Raman and FTIR rely on vibrational modes but selection rules are different (relative band intensities are different) and the influence of aqueous solvent is significant in IR but quite low for Raman. Overall, by applying both techniques it is possible to increase the confidence in a particular estimate of the secondary structure.

VCD provides alternative views of protein conformation with advantages over ECD and FTIR spectroscopy. The important aspect is that VCD, being sensitive to short-range order, senses sheets and other structural elements (including turns) differently than does ECD, which in turn is superior for helix determination. Combination of VDC with ECD and/or FTIR improves determination of all fractional components.

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PROTEIN CHEMISTRY AND MASS SPECTROMETRY IN STRUCTURAL ANALYSIS OF LARGE PROTEINS

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According to the textbook knowledge the use of automated Edman degradation and protein mass spectrometry for the determination of complete primary structure is limited to small proteins with molecular size less than 10 kDa, larger proteins being analyzed by DNA sequencing of the corresponding genes or cDNA clones. Here we present two examples of our recent work in which the sequence of rather large proteins has been determined completely or nearly completely by protein sequencing and mass spectrometry.

The first example includes the pokeweed antiviral protein from *Phytolacca acinosa* (PAP-S) that belongs to the family of type-1 ribosome-inactivating proteins. The purified PAP-S proteins resolve on SDS electrophoresis into two closely related bands with Mr of about 29 kDa. Interestingly, the upper protein band, PAP-S_{up} has been shown to crystallize through carbohydrate-protein interactions based on a rare type of N-glycosylation, namely N-linked GlcNAc monosaccharide substitutions at the canonical Asn-Xxx-Ser/Thr [1]. The sequence of PAP-S_{up} is not known from the genetic data, but is essential for unambiguous solving of the crystal structure in positions that cannot be called directly from the electron density. We have thus determined the complete structure of PAP-S_{up} by Edman degradation of N-terminal and internal peptide sequences in combination with MALDI peptide mapping and tandem mass spectrometry using an ion trap. The complete sequence has 261 amino acids and includes three sites of the above N-glycosylation. The sequence coverage was 92 % by Edman degradation data, 93 % by peptide mapping and 90 % by tandem MS data. The second example is -N-acetylhexosaminidase from *Aspergillus oryzae* CCF1066, a robust extracellular secreted enzyme used in enzymatic syntheses of oligosaccharides and biotechnology [2]. This enzyme has 600 amino acids (including 6 cysteins and 6 sites of N-glycosylation), of which 466 has been verified by direct analysis of the protein (sequence coverage 77 %). Identification of large N-terminal segment in the protein proved difficult pointing to the fast cleavage of this protein segment. Enzyme is composed of cleaved signal peptide, the propeptide sequence involved in regulated secretion, the inactive zincin domain, and the catalytical domain belonging to family 20 of glycohydrolases.

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DYNAMICS OF 1,2-DICHLOROETHANE IN THE ACTIVE SITE OF HALOALKANE DEHALOGENASE LINB: EFFECT OF SOLVENT AND HALIDE ION ON PRODUCTIVE BINDING

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1,2-dichloroethane (DCE) is a toxic and carcinogenic chlorinated compound that is not known to be formed naturally. As many other synthetic halogenated aliphatic compounds, DCE is rather resistant to biodegradation and persists in the environment. Nevertheless, several bacterial cultures that are able to use DCE as the only carbon and halogen source have been isolated. The most efficient catalysis of DCE has been observed with haloalkane dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10. Even lower activity with DCE was observed for haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26. Crystallographic analysis of LinB-DCE complex showed non-productive binding of DCE to the enzyme active site, while molecular docking suggested that DCE molecule can possibly bind to the active site but is prevented by chloride ion and/or water molecules [1].

Two nanoseconds-long trajectories of LinB with different number of ligands bound to the active site were carried out and compared. The results show that productive binding of DCE (i.e., binding to the Michaelis-Menten complex) is blocked by the presence of chloride ion or water molecule in the halide-stabilization pocket of the active site. In case of empty halide-stabilization pocket, the productive binding of DCE occurs very rapidly (in less then 20 ps). On the other hand, DCE locks the chloride ion in the halide-stabilization pocket as was confirmed by steered molecular dynamics simulations and by fact that chloride ion can easily leave LinB active site in the system without DCE.

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MOLECULAR MODELING AS A TOOL IN MOLECULAR BIOLOGY OF MEMBRANE-BOUND RECEPTORS

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The importance of computer modeling of membrane proteins in molecular biology is worked out. We give three examples that models gained by a combined approach of homology and energetic modeling with vibrational spectroscopy are a useful help in site-directed mutagenesis, truncation, binding-studies and even in crystallography. The study of the vanilloid receptor is a successful application of a computer model in the construction of truncations that served for the identification of functionally important protein parts. In the case of CD69 computer docking helped to identify the Ca^{2+}–binding site that was not observed in the crystal structure of this protein due to the non-physiological conditions of crystallisation.

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CDK2 ACTIVATION AND INHIBITION BY PHOSPHORYLATION, A MOLECULAR DYNAMICS STUDY

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In human cell, cell cycle events are governed by several CDKs [1]. Cell-cycle dependent oscillations in CDK activity are induced by complex mechanisms that include binding to positive regulatory subunits and phosphorylation at positive and negative regulatory sites. For activation CDKs require binding to cyclins. CDKs obtain full activity at binding with adenosine triphosphate (ATP) by phosphorylation of a threonine residue in the CDK (Thr 160 in human CDK2) [2]. Activities of these enzymes are inhibited in several ways, for examples, (de)phosphorylation, interaction with various natural protein inhibitors [3]. CDK2 can be negatively regulated by phosphorylation on Tyr15 and to a lesser extent on Thr14 [4].

This work describes behavior of monomeric CDK2/ATP, CDK2/cyclinA/ATP complex, and pT160-CDK2/cyclinA/ATP complex (CDK2/cyclinA/ATP complex phosphorylated on Thr160 residue of CDK2) using the molecular dynamics simulations with the Cornell *et al.* force field as implemented in the AMBER software package [5]. The next MD study was performed on pY15,pT160-CDK2/cyclinA/ATP system. The system was prepared from pT160-CDK2/cyclinA/ATP by phosphorylation of the Tyr15 residue of CDK2. Results of conformational behavior of ATP and key residues for activation in these complexes will be presented. Activation of CDK2 involves various conformational changes, including the reorientation of the phosphate part of ATP and key residues involved in ATP binding site. Transformation of conformation of ATP phosphate in the pT160-CDK2/cyclinA complex is important to form substrate binding site, and is thought to be critical for catalysis.

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APPLICATION OF POWDER DIFFRACTION IN BIOLOGY? THE EGG-SHELL MICROSTRUCTURE

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In last years, renaissance of rather old and traditional technique - X-ray powder diffraction can be observed. This was initiated by both the interest in design of new materials (in materials science, physics and chemistry, where it plays the role of a basic method), and also by fast development in instrumental techniques - X-ray optics and detection which enhanced its possibilities.

Powder diffraction pattern contains different kind of information. Peak positions and intensities are related to crystal (atomic) structure, i.e. the type and size of lattice cell and atomic positions and consequently it can be used for structure refinement and even structure determination in some cases. As a finger print of each individual phase, the diffraction pattern can be an ideal tool for phase analysis.

However, there is much more hidden in the pattern. Variations of lattice parameters and intensitites can detect lattice defects. This is related to the so-called real structure of material, the term which is also used for structural features in the scale of nanometers, i.e. grains or subgrains. The topics which is now of great interest because of intense research of nanomaterials. Complete powder diffraction analysis nowadays includes - phase analysis, structure refinement, stress, strain, crystallite size and texture determination.

Can the technique be of any use for biologists? There have not been many applications yet. Main interest of biologists now seems to be directed to protein crystallography where even synchrotron single crystal diffraction may be insufficient. However, recently, an attempt to use powder diffraction for structure refinement of proteins has appeared [1, 2] too.

In present work, we have tried to perform more complete diffraction analysis of different egg-shells.

The biological function of the egg-shell is a chamber for embryonic development and from which the chick is able to emerge at the appropriate time. The requirements of the table egg industry are different. The industry sustains economic loss from cracked eggs and some of the cracking can be attributed to the deficiencies in the egg-shell structure. This is one of the reasons why the attention to eggshell is devoted [3-5].

The egg-shell consists of several mutually through-growing layers of $CaCO_3$. The innermost layer - mamilary layer (~100 μm) grows on the outer egg membrane and creates the base on which the palisade layer constitutes the thickest part (200 μm) of the egg-shell. The top layer is the vertical layer (5-8 μm) covered by the organic cuticle.

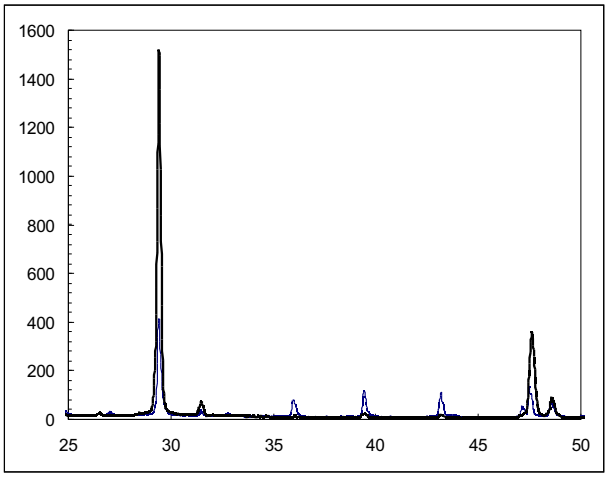
Different kinds of hen´s and bird´s egg-shells in the powder form or as a whole from both sides of the shell were examined by powder diffractometry and film back-reflection method. The powder patterns were evaluated by the fitting of diffraction profiles with the Pearson VII function. The lattice parameters, peak intensities and profile broadening were analysed. At the Bragg-Brentano setting (2 = 40) the Cu radiation penetrates approximately into the 9 μm of the egg-shell, so the measurements from the inner and outer shell surface can give evidence of the mamilary and palisade layer, respectively.

The results obtained on egg-shells of very different origins shown no significant differences in lattice parameters that correspond well to the PDF-2 values. The patterns contained only basic phase $CaCO_3$ (space group no. 167: R-3c) with a small addition of magnesium (0.3 wt. % , determined by atomic absorption). Diffraction patterns of powders obtained from all the eggs investigated correspond very well to the pattern of standard $CaCO_3$. The correspondence is very good including intensities. The patterns obtained from egg-shell powders are also very similar to the standard pattern, regardless larger line broadening.

However, there are differences between powders and both sides of the shells. For inner shell surfaces, the intensities are only slightly different than in powders (including standard one) but there is significant line broadening indicating fluctuations of lattice spacings (the mean local strain of about 0.2 %). On the other hand, for outer shell surfaces, there is much smaller broadening of lines, similar to powders, but significant changes of intensities indicating the 00l textures of grains. This is also an evidence of presence of two basic layers, structurally very different - mamilary and palisade. The meaning of crystallographic texture has been emphasized [3, 4]. It was steted that the breaking strength of the eggshell is inversely related to the degree of calcite orientation and conversely, reduced strength in the eggshell from aged hens coincides with a high variability of texture [3].

As a general conclusion and amazing fact, we can say that any differences of XRD parameters between the eggs of very different origin are not significant. So that their microstructure and composition, as they can be seen by XRD, are the same.

This work was an attempt for non-traditional application of powder diffraction and it was shown that it may be helpful for biologists not only for phase analysis but also for the study of nanostructure of inorganic crystalline phases in biological objects which is closely related to the overall microstructure which is strongly influenced by proteins taking part in the egg creation. The eggshell matrix proteins influences the process of crystal growth by controlling size, shape and orientation of calcite crystals. The formation of avian eggs belongs to most rapid mineralization processes known.



A typical part of the diffraction pattern of the egg-shell ($CaCO_3$) - from the inner (thin line) and outer side (thick line), respectively.

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STRUCTURAL AND DYNAMIC STUDIES OF THE 12 KDA FORM OF PROTEASE FROM MASON-PFIZER MONKEY VIRUS

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Mason-Pfizer monkey virus encodes an aspartic protease (M-PMV PR), which is essential for the correct assembly and maturation of the virion particles. The protease processes viral protein precursors yielding fully functional structural proteins and enzymes. It was demonstrated that the enzyme exists in three active forms with molecular mass of 17, 13, and 12 kDa per monomer, which makes M-PMV PR quite unique among other retroviral proteases [1].

We will report a complete three-dimensional structure of the shortest form of the protease (12 kDa) where both cysteine residues (Cys7 and Cys106) were mutated for alanines to prevent their oxidation [2] and the activity of the protease was suppressed by an exchange of the catalytic aspartate for asparigine in the position 26. Doubly labeled ($^{13}C/^{15}N$) sample was prepared and the resonance assignment was based on triple resonance multidimensional NMR experiments [3]. Based on the calculation of chemical shift index (CSI) approximate positions of secondary structure elements were located. The refinement of the structure was carried out by ARIA software package [4] based on NOE contacts, dihedral angle restraints and hydrogen bonds. To support the structural results we also measured ^{15}N relaxation properties of M-PMV PR to obtain a picture of dynamic behavior of the protein.

It turned out that the lower activity of the shortest form of the protease, as compared with the fully active 17 kDa form, is caused by the prevailing monomer in solution. This result was supported by ultracentrifugation experiments. We have proved that the monomeric form of the 12 kDa M-PMV PR is folded similarly as the other retroviral proteases with several distinct features, which will be discussed.

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NMR RELAXATION STUDIES OF FAST INTERNAL MOTIONS IN NUCLEIC ACIDS

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Investigations of intramolecular dynamics on the nanosecond to picosecond time scale by solution NMR spectroscopy are based on the spin relaxation properties of nuclei such as ^{15}N or ^{13}C. The most commonly measured quantities are the longitudinal relaxation rate constant, R_1, the transverse relaxation rate constant, R_2, and the steady state nuclear Overhauser effect (NOE). The values of these experimentally obtained parameters can be expressed as linear combinations of spectral density functions. The spectral density function characterizes the overall rotational diffusion of the molecule as well as its intramolecular motions. Two approaches exist for the interpretation of the experimental data. In spectral density mapping, the values of the spectral density function J( ) at characteristic frequencies are determined from the relaxation data [1]. The "model-free" formalism assumes a particular form of the spectral density function, whose parameters then characterize amplitudes and time scales of the molecular motions [2].

The methods for measuring ^{15}N relaxation parameters have been well established thanks to a large body of literature on protein dynamics studied through amide nitrogen relaxation. Because of the similarity of the spin environments, the procedures developed for protein amide nitrogen are directly applicable to imino nitrogen of guanine and uracil [3]. However, ^{15}N relaxation study of nucleic acids can yield the dynamic properties of guanine and uracil bases only. For a more complete picture, the use of ^{13}C relaxation data is highly desirable. The most suitable candidates for relaxation studies in nucleic acids are C8 carbons of purines, C6 carbons of pyrimidines and C1' of the sugar.

In order to bring insight into the internal dynamics of RNA tetraloops we have performed a ^{13}C NMR relaxation and molecular dynamics study of 14-nt RNA hairpins GGCACUUCGGUGCC and GGCACGCAAGUGCC (the underlined nucleotides form the loops). The UNCG and GNRA families of stable RNA hairpins (where N is any nucleotide and R is purine) have very similar overall folds. However, the biological roles of these two sequences appear different. The differences have been attributed to distinct dynamical properties of the two sequences [4].

We have measured R_1 and $R_{1\rho}$ relaxation rates for C8 of purines, C2 of adenines, C6 and C5 of pyrimidines as well as for C1' of the ribose sugars at several magnetic field strengths. The data have been interpreted in the framework of modelfree analysis characterizing the internal dynamics of the molecules by order parameters and correlation times for fast motions on the picosecond to nanosecond time scale and by contributions of chemical exchange.

While both tetraloops exhibit increased mobility on the fast time scales, with the GCAA loop we have detected a significant contribution of conformational dynamics on the millisecond to microsecond time scale. This is consistent with the observations that the GNRA family appears more

flexible and tolerant of the conformational changes important for molecular recognition.

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CONFORMATIONAL VARIABILITY OF RNA BACKBONE

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As shown by ribozyme and especially ribosome structures solved in last few years, molecules of RNA form complicated 3D folds which have no match among known DNA structures but their complexity is quite comparable to that of protein folds. Complicated RNA folds are enabled by a high flexibility of the nucleotide backbone but little is known about its conformational behavior. A well refined structure of the large ribosome subunit 50S at 2.4A, NDB structure RR0033 (PDB ID 1JJ2), Ban *et al.* Science **289**, 905 (2000), provides a database of over 2700 nucleotides. This work analyzes conformations of these nucleotides by a combination of Fourier averaging and clustering techniques.

Majority of all nucleotides of RR0033, about 70%, are in the A-type conformation, this main conformational type can be further classified into three subclasses. The remaining 30% of nucleotides with other than A-type conformations were analyzed in a greater detail. The backbone torsion angles for each nucleotide were grouped into eight sets of three angles with the main emphasis on the torsions around the two phosphodiester bonds, O3*-P (torsion zeta) and P-O5* (alpha). Each set of three torsions results in a 3D distribution of points in a parametric torsional space and this distributions was Fourier transformed into densities of nucleotide conformations. Peak positions (maxima) of these maps confine the most probable (di)nucleotide conformations.

Nucleotides belonging to the same peaks in several torsional 3D maps have similar geometry. Such nucleotides were grouped and compared in Cartesian (real) 3D space. In such a way, twelwe types of highly untypical (non-A) nucleotide conformations were identified and their Cartesian coordinates determined. These untypical nucleotide conformations can be useful in e.g.refinement process and are available upon request.

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STRUCTURE AND DYNAMICS OF RIBOSOMAL 5S RNA AND ITS COMPLEX WITH RIBOSOMAL PROTEIN L25

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Ribosomal 5S RNA (5S rRNA) is an integral component of the large ribosomal subunit in all known organisms with the exception of the small ribosomes of fungal and animal mitochondria. The 5S rRNA of *Escherichia coli* (*E. coli*) interacts with ribosomal proteins L5, L18 and L25 and enhances protein synthesis by stabilization of the ribosome structure but its exact role in protein synthesis is still not known. 5S rRNA contains internal loop - **Loop E**. The Loop E is a salient example of a uniquely structured non-Watson-Crick motif, as it contains seven consecutive non-Watson-Crick base pairs, including wobble G.U base pair and substantial cross-strand purine stacking. This unique duplex architecture together with adjacent sequence helix IV form binding site for ribosomal protein L25.

To understand the structure and function of internal Loop E and interaction between 5S rRNA Loop E and ribosomal protein L25, we have carried out set of molecular dynamics simulations.

Initial structures were directly taken from x-ray crystallography - crystal structure of 5S rRNA Loop E (*E. coli*) [1] and crystal structure of ribosomal protein L25 complexed with the 5S rRNA fragment [2]. Another studied structure was chloroplast Loop E for which there is no atomic resolution structure yet available and which is sufficiently different from bacterial Loop E motifs in sequence, but evolutionarily related to it. Model of chloroplast Loop E was proposed based on homology modeling [3], initial structure for this model was bacterial Loop E, mutation of three base pairs was performed based on the isosteric mutation.

Main focus of our investigation was to study of the structure, dynamics, hydration and cation binding of non-Watson-Crick base pairs and interaction between ribosomal protein L25 and 5S rRNA Loop E. Another aim of this study was to test the usefulness of the MD technique in

evaluating the dynamics and energetics of molecular models of RNA motifs constructed by phylogenetic analysis and isostericity principles.

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THE EFFECT OF WATER SOLVENT ON THEORETICAL NMR SPIN - SPIN COUPLINGS IN DNA: IMPROVEMENT OF CALCULATED VALUES BY APPLICATION OF TWO SOLVENT MODELS

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The NMR indirect spin - spin coupling constants calculated in DNA base pairs are compared with one-bond $^1J(X,H)$, $^1J(C,X)$ and two-bond 2J(X,H), (X = C, N) coupling constants measured in DNA hairpin molecule d(GCGAAGC) [1]. The two theoretical models of solvent: explicit and Polarized Continuum, were introduced. Both explicit and PCM solvent model provide a similar improvement of the calculated J constants when compared to the experimental data. The mean absolute deviation between the calculated and experimental couplings is significantly reduced by solvent inclusion, from 1.7 to 1.1 Hz for guanine, from 2.4 to 0.6 Hz for cytosine, and from 2.3 to 1.6 Hz for adenine. The strongest solvent shift was calculated for the $^1J(C,H)$ coupling constants, particularly for the (C8,H8) coupling in guanine (6.1 Hz) and adenine, and the (C5,H5) and (C6,H6) couplings in cytosine. These changes in $^1J(C,H)$ coupling seem to correspond to the charge transfer from water bonding and lone pair orbitals to the guanine anti-bonding C8-H8, C8-N7, and C8-N9 orbitals. From the close agreement between the calculated and experimental coupling constants we can deduce that the C-H group of the hairpin bases is extensively hydrated even when interaction with solvent is rather weak and non - specific.

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LECTURES - MARCH 15

STRUCTURAL BASIS OF HIV-1 AND HIV-2 PROTEASE INHIBITION BY A MONOCLONAL ANTIBODY

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Since the demonstration that the Human Immunodeficiency Virus protease (HIV PR) is essential in the viral life cycle [1], this enzyme has become one of the primary targets for antiviral drug design. With the objective of probing the structural stability of HIV PR and the eventual design of potential inhibitors directed to regions other than the active site, we have examined the effects of anti-HIV-1 PR monoclonal antibodies (mAbs) on the catalytic activity of the protease [2, 3]. Monoclonal antibody mAb1696, although raised against the HIV-1 PR, inhibits the catalytic activity of both the HIV-1 and HIV-2 enzymes with inhibition constants in nanomolar range [3] and cross-reacts with peptides comprising the N-terminus of the HIV protease (residues 1 to 7). The N-terminal region is essential for dimerization of monomers and thus forming the active HIV PR dimer.

To investigate further the mechanism of HIV PR inhibition by mAb1696, a recombinant single-chain Fv fragment (scFv) that contains heavy and light chain variable domains of mAb1696 joined by a flexible linker have been prepared and used for crystallographic studies [4]. Although the crystallization of scFv1696 complexed with the HIV proteases has not yet been successful, the formation of these complexes has been demonstrated in solution. As an alternative approach to studying the antigenic recognition by the mAb 1696 at the structural level, we have crystallized complexes of scFv1696 with the respective epitope peptide fragments of the HIV protease. The three-dimensional structure of the complex formed between scFv1696 and epitope peptide PQITLWQRR (corresponding to the N-terminus of HIV-1 PR) has been solved and refined at 2.70 Å resolution [4]. The structure of the complex formed between scFv1696 and epitope peptide PQFSLWKR (corresponding to the N-terminus of HIV-2 PR) has been solved and refined at 1.88Å resolution.

Interactions of peptides with scFv1696 have been analyzed and compared in both structures. On the basis of the interactions seen in the complex, the cross-reactivity between mAb1696 and the HIV-1 and HIV-2 protease and their N-terminal peptides can be explained. The broad reactivity between mAb 1696 on one side and the HIV-1 PR and HIV-2 PR derives from the invariance or conservation

of the first six N-terminal residues of the two strains. Most of these residues are deeply buried in the antibody-binding groove and establish extensive contacts.

Using the peptide as a guide, a docking complex of a whole protease monomer was generated, which suggests that mAb 1696 inhibits the HIV PR by favouring the dissociation of the active homodimer. A dissociative mechanism of protease inhibition by 1696 is consistent with the stoichiometry of the inhibition complex, as derived from the inhibition kinetic studies [3].

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CRYSTALLOGRAPHIC STUDY OF AN ANTI -CARBONIC ANHYDRASE IX MONOCLONAL ANTIBODY M75

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Carbonic anhydrase IX (CA IX) is a cell surface protein, strongly associated with certain types of human carcinomas. The predicted protein of cloned CA IX cDNA consists of the signal peptide, proteoglycan-related sequence, carbonic anhydrase domain, trasmembrane segment and a short intracellular tail (1, 2). Until now, molecular basis of involvement of CA IX in carcinogenesis has remained unclear. CA IX is a cell adhesion molecule, its carbonic anhydrase (CA) is enzymaticaly active. Structural study of a CA IX-binding monoclonal antibody (mAb) M75, complexed with its epitope peptide may contribute toward elucidation of the role of CA IX. To achieve this goal, two parallel approaches were chosen: analysis of Fab fragment, or of a smaller scFv fragment, both containing the complete antigen binding site present in mAb M75.

Monoclonal antibody M75 was obtained (3) and proved to react excellently with native and denaturated CA IX. Using synthetic oligopeptides, the epitope of mAb M75 was localized in the proteoglycan domain of CA IX, in the region of a tandem repeat and identified as amino acids PGEEDLP (4). The Fab fragment was obtained by papain cleavage. We obtained crystals of free Fab M75 and Fab M75 complexed with two different epitope peptides. The data set for Fab M75 was collected and the structure solving is underway.

Another approach is to prepare sc Fv fragment of this antibody (described in the contribution of Vlastimil Král, Milan Fábry, Magda Hořejší, Jan Zavada, Juraj Sedláček: Molecular cloning, *E. coli* expression and purification of scFv antibody fragments of diagnostic/therapeutic interest.

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A LONG WAY TO WELL DIFFRACTING PROTEIN CRYSTALS

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In experiments of protein crystallization very often spherulites, microcrystals and needles appear as a result of not having optimal crystallization conditions. Optimization of conditions is often guided by a sense of protein, however, theoretical knowledge and practical experiences are inevitable. It is well known that the best conditions for growing crystals differ from crystal nucleation conditions. Separation of these two processes in order to obtain well diffracting crystals will be presented. The importance of protein purity and homogeneity in growing crystals will be stressed and procedures improving crystal quality will be discussed. One of the topics will concern practical aspects for preparation of protein crystals for data collection at room and cryogenic temperature. All these points will be documented by our experiences with crystallization of a number of proteins, their mutants and complexes.

PRECISION AND RELIABILITY IN MOLECULAR STRUCTURE DETERMINATION

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Protein structure database (PDB) [1] is a primary source of information about the structure of biological macromolecules. It contains almost 20.000 of experimentally determined structures. About 15 % of them are determined by NMR techniques, 85 % by X-ray crystallography (see Tab.1).

In spite of the fact that the final calculations and refinement of the structure with NMR and X-ray data can be performed with the same computer program (e.g. XPLOR, CNS [2]) there are significant differences in meaning and also in presentation of structure.

NMR measurement. Roughly speaking, the most important information from NMR measurement is identification of atoms laying near (3 - 6 Å) each other in spite of the fact that they are far along the chain. The final description structure is obtained by searching for all molecular models satisfying these experimental restrains using the methods of molecular modelling. Thus, generally speaking, the reliability of the structure model is derived from a completeness of the experimental restrains, and the exact atom coordinates are optimized by methods of molecular modelling. The molecular structure in PDB is described as a number of individual structures often interpreted as snapshots of a molecule in movement.

X-ray diffraction experiment. The primary result of X-ray diffraction experiment is a map of electron density averaged over time of measurement and all structure units in crystal. However, it is really never published in this form. The atomic coordinates send to the PDB are determined as centers of electron density of individual atoms. Moving parts of molecule correspond to areas with low or smashed electron density. At this moment, the X-ray scientist starts to look for several alternative conformations which are all refined under the restrain that the sum of occupation factors is 1. Thus the information about molecular movement is hidden in a single file of atom coordinates as alternative conformations for individual side chains and also as temperature factors B [3] describing the mean atomic displacement u(Å) around the mean positions of individual atoms . The dependence of B on the mean atomic displacement u (Å) is ilustrated in Tab. 2.

Another term sometimes misunderstood is resolution. The precision of atomic positions is not a simple function of resolution and depends on more factors. An approximate relation between the expected standard deviation of atomic position and the resolution is illustrated in Tab.3.

The talk will show complementarity of X-ray and NMR techniques and some rules for working with data obtained by X-ray crystallography.

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Table 1. Number of structures of biological macromolecules deposited in the Protein structure database. The theoretical models are not being collected in the PDB since 2002 *(information taken from the PDB Holdings List: 04-Feb-2003)*

	Proteins Viruses	Protein/NA Complexes	Nucleic Acids	Carbohydrates	Details of measurement	total
X-ray	15.507	734	638	14	8.755(i)	16.893
NMR	2.481	89	496	4	1.457(ii)	3.070
Total	17.988	823	1.134	18	10.212	19.963

(i) Deposition of reflection intensities, (ii) Deposition of restrains gained by NMR measurement

Table 2. Exact relation between the temperature factor B (Å^2) and the effective atom width (the mean atomic displacement u (Å)). $B = 8\ ^2\langle u^2\rangle$.

B (Å)	4	8	16	32	64	128
Mean atomic displacement (Å)	0.23	0.32	0.45	0.64	0.90	1.80

Table 3. Typical average coordinate inaccuracy $\langle\ _x\rangle$ (mean expected standard deviation) as a function of the limit for diffraction measurement (resolution). Data collected from randomly selected structures found in literature.

Resolution (Å)	5	3.0	2.4	1.9	1.5	1.3	1.0	0.8	0.6
Expected e.s.d. (Å)	> 3	0.7	0.4	0.2	0.1	0.07	0.05	0.03	0.01

THE CRYSTAL STRUCTURE OF YODA, AN *E. COLI* PROTEIN INVOLVED IN HEAVY METAL STRESS

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Heavy metals, such as mercury and cadmium, are very toxic in living organisms, which have therefore evolved various defence and control mechanisms. Even for metals that are essential for the correct functioning of living organisms, their presence within the cells has to be tightly controlled to avoid negative effects. In most cases, and certainly in the case of cadmium, the actual toxic effect is in part due to the oxidant properties of these metals.

We have solved the structure of YodA, a novel protein implicated in cadmium stress is *E. coli*. This protein has been suggested as a member of a new family of cadmium-response proteins in bacteria (1). While there is no sequence similarity to proteins with known folds, the three-dimensional structure shows that YodA is a memeber of the lipocalin/calycin family. At the same time, we show that YodA is a metalloprotein, with a high-affinity site for divalent cations such as zinc, nickel and cadmium.

We shall describe the structure of the protein and propose hypotheses for its function in bacteria.

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STRUCTURAL BIOLOGY OF 14-3-3 PROTEINS

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14-3-3 proteins were the first signaling proteins to be identified as discrete phosphoserine/phosphothreonine binding molecules. These proteins play an important role in the regulation of signal transduction, apoptosis, cell cycle control, and nutrient-sensing pathways [1,2]. The 14-3-3 proteins are a conserved family of acidic proteins (molecular mass ranging from 27 to 32 kDa) present in high abundance in all eukaryotic organisms studied so far. Many organisms express multiple isoforms; for example, in mammals seven isoforms have been identified. All 14-3-3 isoforms can form stable homo and hetero-dimers. Though 14-3-3 proteins perform different functions for different ligands, general mechanisms of 14-3-3 action include changes in activity of bound enzymes, control in sub-cellular localization of 14-3-3 bound proteins, and alterations in protein-protein interactions of bound ligands with other proteins.

Crystal structures of human 14-3-3 zeta and tau isoforms, and structures of 14-3-3zeta bound to various peptides representing 14-3-3 binding motifs provided first structural insight into understanding of the biological function of 14-3-3 proteins [3, 4]. These structures illustrate the conserved fold of the 14-3-3 proteins, where each monomer is composed of nine antiparallel -helices, and two monomers form cup-shaped dimers with a large deep channel in the center running the length of the dimer. The walls of the channel contain amphipathic grooves that are ~30 Å long, and residues lining the grooves are mostly conserved among the different isoforms. Phosphoserine-containing peptides were observed to bind in an extended conformation within these grooves. Recently, the structure of 14-3-3zeta bound to an enzyme serotonin N-acetyltransferase in complex with a bisubstrate analog, was solved [5]. This structure allowed to describe how 14-3-3 interacts with an enzymatically active protein - 14-3-3 stabilizes the conformation of an adjacent region in the enzyme, causing enhanced substrate binding and product formation.

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STRUCTURE OF THE PLECTIN ACTIN BINDING DOMAIN

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Plectin and its isoforms are versatile cytolinker proteins of very large size (molecular mass over 500 kDa) that are expressed in a wide variety of mammalian tissues and cell types. Biochemical data indicate that plectin plays an important role in cytoskeleton network organization and regulation, with consequences for viscoelastic properties of the cytoplasm and the mechanical integrity and resistance of cells and tissues. Defects in plectin genes cause autosomal recessive or dominant hereditary diseases, characterized by severe skin blistering with or without muscular dystrophy. Plectin has been well characterized biochemically and genetically. Electron microscopy revealed that the protein has a dumbbell-like structure comprising a central rod domain (approximately 2 000 Å long) flanked by N- and C- terminal globular domains. Each of these domains contains several subdomains to which binding sites for various interaction partners have been mapped. Actin-binding domain (ABD) of plectin is located in proximity to its N-terminus. It consists of two so called calponin homology (CH) 1 and 2 subdomains.

Crystals of the plectin ABD were grown by the hanging drop diffusion method. Modification of crystallization conditions resulted in two crystal forms. Data from crystal form I (P21) were collected at room temperature to 2.0 Å resolution and from crystal form II (P212121) at cryo temperature to 2.2 Å resolution on the EMBL beamlines at the DORIS storage ring, DESY Hamburg. The structure was solved by molecular replacement method using utrophin ABD (PDB code 1QAG) as search model. Structures of both crystal forms were refined with the program REFMAC5. Recombinant molecule of the plectin ABD is a protein consisting of 245 residues which form 11 helices. The structure is almost identical with the fimbrin ABD in spite of relatively low amino-acid sequence identity (23 %) and differs from those of utrophin and dystrophin mainly in orientation of CH1 and CH2 subdomains.

POSTERS

MOLECULAR DYNAMICS SIMULATION OF 1,2,3-TRICHLOROPROPANE IN THE ACTIVE SITE OF WILD TYPE AND MUTANT HALOALKANE DEHALOGENASE DHAA

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1,2,3-trichloropropane (TCP) is a toxic synthetic chlorinated hydrocarbon not known to occur naturally. TCP is resistant to biological and chemical degradation and is often found as a water pollutant. Thermodynamics calculation shows that aerobic mineralization of TCP could provide sufficient energy to sustain microbial growth. The haloalkane dehalogenase (DhaA) from *Rhodococcus sp*. m15-3 hydrolyzes carbon-halogen bonds in a wide range of haloalkanes, including TCP, to the corresponding (halo)alcohol, releasing halide ions. Recently a way how to improve DhaA enzyme to utilize TCP as a substrate by double-point mutation (C176Y+Y273F) has been proposed by Bosma *et al*. [1].

The goal of the work was to explain by molecular modeling why is the mutated enzyme more effective than wild type DhaA. The molecular dynamics method was used to produce six 1 ns-long simulations; three of them with the DhaA wild-type in complex with TCP in tree different binding modes TCP(bm1), TCP(bm2), and TCP(bm3) and three of them with C176Y+Y273F double mutant of DhaA. The binding mode TCP(bm1) corresponds to dehalogenation from C atom, while the binding modes TCP(bm2) and TCP(bm3) relates to the dehalogenation from either C atom.

Both simulations of wt/TCP(bm1) and C176Y+ Y273F/TCP(bm1) show that the dehalogenation from C is not possible, due to a sterical hindrance of TCP in the active site. Moreover, TCP changes its binding mode from TCP(bm1) to TCP(bm2) in both these simulations. The other simulations (wt /TCP(bm2), wt /TCP(bm3), C176Y+ Y273F/TCP(bm2) and C176Y+ Y273F/ TCP(bm3)) show that TCP frequently adopts a near attack conformation (NAC), i.e. conformation appropriate for S_N2 attack during the whole simulation. Preliminary results indicate that NAC is more populated in simulation of double mutant (see Table 1).

Table 1: Population of NAC for studied systems.

System	TCP(bm2)	TCP(bm3)
wt-DhaA	20.7 %	6.5 %
C176Y+Y273F-DhaA	28.9 %	11.7 %

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MOLECULAR DYNAMICS SIMULATIONS OF DNA TRIPLEXES CONTAINING MODIFIED HOOGSTEEN STRANDS - POTENTIAL CANDIDATES FOR ANTIGENE THERAPY

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The concept of "antisense" and "antigene" nucleic acids represents a perspective approach in chemotherapy, promising to inhibit selectively unwanted gene expression by creation of a helical complex with target mRNA or DNA (carrying "sense" genetic information) [1]. The oligonucleotides with natural chemical composition have been, however, found as unsuitable for in vivo applications because of their insufficient resistance against nucleases. That is why numerous novel-type nucleotide analogs are designed, synthesized and tested [1-6].

A number of phosphonate-based mononucleotide analogs containing an O-(phosphono)methyl group instead of the natural phosphonomonoester one were found to be potent antivirals: this indicated enzyme stability of the phosphonate -O-P-CH_2-O- bond [7]. Several types of isopolar modified oligothymidylates and oligoadenylates (15 mers) with the phosphonate -O-P-CH_2-O- internucleotide linkage were prepared. The modified oligonucleotides were subjected to the study of their hybridization properties, resistance against nucleases, and the ability to elicit RNase H activity [2]. Impact of the internucleoside linkage modification by inserting a methylene group on the ability of the modified oligonucleotide to hybridize with a natural DNA and RNA strand was studied by fully solvated molecular dynamics (MD) simulations [3-6].

Triplex forming oligodeoxynucleotides have attracted a great deal of attention because of their potential use in gene therapy. In inter molecular triplexes, third strand of ODN binds to the major groove of the DNA. However, in general, the binding of a third-strand ODN to a target DNA duplex is thermodynamically weaker than duplex formation itself. Thus much effort has been made to increase the affinity of the third strand for its target. ODN analogues carrying various aminoalkyl linkers have been synthesized, some of which have been shown to increase the thermal stability of triplexes [8]. The thermal stabilization can be explained by an electrostatic interaction between the positively charged aminoalkyl residue of the nucleosides and a pro-R oxygen of a negatively charged phosphate at the second strand of the target DNA.

The present work deals with the phosphonate analog of the natural phosphodiester internucleoside linkage in conjunction with various aminoalkyl-linkers. Several triple helical structures consisting of a natural Watson-Crick duplex and a modified Hoogsteen thymidine strand were used as model systems. Impact of the sugar phosphate backbone modifications on the ability of the modified oligonucleotides to hybridize with a nautral duplex, was studied by molecular dynamics simulations. The nucleic acids were surrounded by a periodic box of ~10000 TIP3P water atoms. Fully solvated trajectories were computed using the AMBER 5.0 software package. The implemented force field doesn't contain force constants needed to describe the modified parts of the phosphonate analogs [9]. The completion was made on the base of ab initio calculations [3].

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FUNCTIONAL RECONSTITUTION OF PHOTOSYSTEM 2 INTO LIPOSOMES

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Most recent structural data on photosystem 2 (PS2), the first membrane protein complex in the photosynthetic electron transport chain, confirm that this complex exists as a functional dimer in the thylakoid membrane of cyanobacteria [1, 2]. Besides the membrane embedded part of this dimer with dimensions of 190 Å x 100 Å x 40 Å, this complex also extends about 10 Å out of the membrane in the stromal region and 55 Å in the lumen; the latter is referred to as the oxygen evolving complex, harbouring the water-splitting site. Developing an approriate method to reconstitute dimeric PS2 into liposomes should finally help

to answer the fundamental question concerning its structure-based function: Is a dimeric structure a prerequisite for optimal water-splitting activity (monomeric complexes, solubilized by detergent, are active, too, although at a lower level) and which is the impact of the lipid-phase-composition on the water-splitting activity? Here we present data on the orientation of reconstituted dimeric PS2 from the cyanobacterium *Thermosynechococcus elongatus* and also give indications for its activity within the liposomes and monomer-dimer distribution (by EM analysis).

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THEORETICAL MODEL OF CU(I)/CU(II) HYDRATION UNDER THE INFLUENCE OF VARIABLE LIGAND FIELD

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An ab initio second-order perturbation theory (MP2) and DFT calculation were performed on the copper monovalent and divalent cations. Different number of the water molecules was considered for the pure $Cu(H_2O)_n^m$ ions where n = (1 to 6), m = 1 or 2. In the next step, copper complexes with four and six ligands were regarded where the number of the ammonium molecules was varied. As a final step complexes of Cu-guanine with 5 water molecules were calculated.

Pure copper hydration clearly shows that while Cu(II) complexes are relatively good stabilized up to coordination with six water molecules, the Cu(I) cation prefers only two-water coordination when electron-correlation effects are included. The other water molecules leave to second hydration sphere. Some interesting theoretical consequences appear when only Hartree-Fock level is taken into account.

Under the ammonium surrounding, the individual stabilities are changed but all qualitative conclusions found for pure water complexes remain valid. Also, some small changes in geometries are apparent as a consequence of different ligand strength of the ammonium molecule.

A slightly stronger ligand - guanine in N7 site also prefers two coordinated Cu(I) complex with the other water escaped from first hydration sphere of Cu cation. Interesting feature, from the structural point of view, can be regarded as the position where the other water molecules are placed in comparison with the hydration of the plain guanine molecule. On the contrary, hydrated complex Cu(II)-guanine exhibits the largest stabilization among all the studied Cu complexes.

ELECTRON MICROSCOPY AND SINGLE PARTICLE ANALYSIS OF PHOTOSYSTEM II FROM RED ALGA *PORPHYRIDIUM CRUENTUM*

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Photosystem II (PSII) is a multisubunit pigment-protein complex embedded in the thylakoid membranes of higher plants, algae and cyanobacteria [1-3]. It performs series of photochemical reactions resulting in the reduction of plastoquinone, the oxidation of water, and the formation of a transmembrane pH gradient. The essential components of the PSII complex are intrinsic membrane proteins that are almost identical between cyanobacteria and higher plants: they include the D1 and D2 reaction center proteins, chlorophyll *a*-binding proteins CP47 and CP43, and subunits of cytochrome *b-559* (cyt *b-559*) and several low-molecular weight proteins with unknown functions [3, 4].

In addition, there are extrinsic proteins associated with PSII, which play important roles in maintaining the function and stability of the oxygen-evolving complex [5]. As both cyanobacteria and higher plants contain 33-kDa extrinsic subunit they differ in composition of the other lumenal subunits. While higher plants and green algae contain the 23 and 16 kDa extrinsic subunits, in cyanobacteria, these proteins are replaced by another two proteins coded by *psbU* and *psbV* gene (cyt c_{550} and 12-kDa subunit) [6]. Red algal PSII complex has a 20 kDa protein in addition to the three cyanobacterial proteins [7]. Recently, structure of the PSII complex isolated from two cyanobacterial strains has been presented [8,9]. These models give the idea of the arrangement of the intrinsic and extrinsic subunits. However, a location of the 20 kDa extrinsic subunit within the red algal PSII is still unknown.

I this report we present preliminary results on locating the the 20 kDa extrinsic protein using a transmission electron microscopy and single particle image analysis of nega-

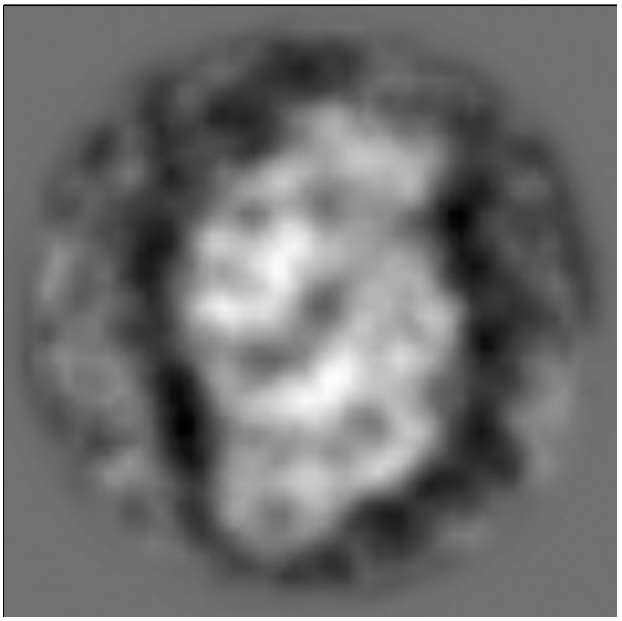

Fig. 1. Averaged projection of negatively-stained preparation of the dimeric PSII complex isolated from Porhyridium cruentum in its top-view projection (i.e. perpendicular view to the thylakoid membrane).

tively-stained preparations of Photosystem II isolated from a red alga *Porhyridium cruentum*.

The PSII complex was isolated from thylakoid membranes from *P. cruentum*. Sucrose density gradient centrifugation of thylakoid membranes solubilized with -dodecylmaltoside resulted in the separation of three green bands. On the basis of protein composition, absorption and 77K fluorescence emmision spectra, the lowest green band was used for further isolation of the PSII complex. This crude PSII extract were solubilized with ß-dodecylmaltoside and loaded on DEAE-Sepharose CL-6B column according to [7]. The purified PSII complexes were eluted with 200 mM NaCl and analyzed.

Electron microscopy was performed with a Philips TEM 420 at magnification of 60,000 . The PSII complexes were applied on the glow discharged carbon-coated copper grids and negatively stained with 2% uranyl acetate. The image analysis was carried out with SPIDER software according to [10]. A total number of 253 single particle top-view projections were extracted from 14 negatively-stained electron microscopy images. The averaged top-view projection of PSII complex indicated a trapezoid particle with a dimension of 21 13 nm (Fig 1). Although no symmetry has been imposed during the image analysis clearly two-fold rotational symmetry around the center of the complex is visible indicating the dimeric nature of the PSII complex. This result is consistent with similar PSII preparations from both cyanobacteria and higher plants suggesting the PSII complex is structurally very similar in organism performing oxygenic photosynthesis [8, 9, 11 - 13].

In order to investigate the organization of the extrinsic subunits (and mainly the location of 20 kDa protein) the PSII particles will be exposed to various salt treatments which remove the lumenal subunits. Thus, a difference map of averaged projections of PSII complexes with and without the extrinsic subunits would enable to determine the location of the 20 kDa subunit.

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CRYSTALLIZATION OF KILLER PROTEIN SPOIISA AND ITS ANTIDOTE SPOIISB FROM *BACILLUS SUBTILIS*

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The *B. subtilis spoIISA* gene encodes a 248-residue protein containing three predicted transmembrane domains [2] with the last two-thirds of protein being located in the cytoplasm. The *spoIISB* gene codes for a hydrophilic 56-residue protein. None of these proteins shares any sequentional similarity to a protein of known function, providing no clue to their function and evolutionary origin. The *spoIISB* translation start codon overlaps the *spoIISA* translation stop codon what is a strong indication that the two genes constitute an operon [1].

A null mutation in *spoIISB* leads to the strong sporulation defect, whereas disruption of either *spoIISA* or whole *spoIIS* locus has no effect on sporulation. Altogether, these facts indicates that a) *SpoIISA* prevents normal progression of the sporulation process; b) *SpoIISB* neutralizes the action of *SpoIISA*; and c) *spoIIS* locus does not play essential role in sporulation process. The strain carrying the *spoIISB* null allele does not exhibit any obvious defect during exponential growth. This immunity of exponentially growing cells to the absence of *SpoIISB* most likely reflects the existence of a threshold concentration below which *SpoIISA* does not significantly impair cell viability, since the induced expression of additional *spoIISA* gene copy led to rapid drop in optical density of exponential phase cell [1].

Since it has structural features of an integral membrane protein, *SpoIISA* could act as a holin and allow some endolysin to gain access to the peptidoglycan [3]. Local solubilization of the cell wall would lead to membrane disruption and consequently to the large plasmolysis zones which were observed by electron microscopy [1]. However, *SpoIISA* does not show any similarity to known holins and is significantly larger than holins identified so far [3]. It is therefore quite possible that the cytoplasmic membrane itself is the target of the toxic action of *SpoIISA*.

In our work we over-expressed cytosolic part of *SpoIISA* His-tag fusion protein together with intact *SpoIISB* protein in *Escherichia coli*. The both proteins were purified using single step metal chelate affinity chromatography, and therefore isolated proteins formed stable complex, which indicates their specific interactions. The gel filtration and electrophoresis experiments showed that the most abundant form of the complex is oligomer consisting of two SpoIISA and two SpoIISB molecules. This observation confirms the results gained using genetic complementation experiments, which predicted that SpoIIA acts as an oligomer [1]. The purified SpoIISA-SpoIISB protein complex was used for crystallization trials.

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SHORTCUTS TO MUSHROOMS: NMR AND MS ANALYSES OF FUNGAL PYRANOSE OXIDASE

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Pyranose 2-oxidase (P2O, EC 1.1.3.10), a fungal periplasmic homotetrameric flavoprotein (~300 kDa), has received increased attention due to its potential analytical and biotechnological applications [1]. This enzyme catalyzes C-2/C-3 oxidation of numerous sugars to their corresponding dicarbonyl derivatives (aldos-2-uloses or glycosid-3-uloses), accompanied with the reduction of flavin adenine dinucleotide (FAD), an obligatory cofactor. P2O has a great biotechnological potential as a catalyst in the key step of C-2 oxidation of D-glucose and D-galactose in the production of modern low-caloric sweeteners D-fructose and D-tagatose.

Our research on the fungal pyranose oxidase followed three subjects. The first one was the study of the enzyme substrate specificity and characterization of its reaction products by spectral analyses (NMR, FAB mass spectrometry) [2,3]. Further, we applied MALDI mass spectrometry with post-source decay (PSD) analysis to determine sequence segments suitable for designing PCR primers for cloning cDNA corresponding to the P2O gene [4]. Finally, we elucidated the structure of the P2O flavin-binding domain, which is of importance for understanding the enzyme reaction mechanisms and possible optimized application. The combination of PSD-MALDI MS and electrospray ion trap mass spectrometry (ESI IT-MS) on the isolated flavopeptide identified flavopeptide sequence, flavin type and flavin linkage site. The type of the aminoacyl flavin covalent link was determined by NMR spectroscopy resulting in the structure STXW with X = 8 -(N^3-histidyl)-FAD [5].

The work was supported by Program for Scientific-Technical Cooperation AKTION, Austria-Czech Republic (2002-9) and Institutional Research Concept AV0Z5020903.

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RECONSTITUTION OF MEMBRANE PROTEIN PSBH INTO NATURAL ALGAL LIPIDS

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Study of membrane proteins in their native environment is restricted from the complexity of native membranes, interference with other membrane constituents and other reactions. To understand organization of the biological membranes and the interaction-taking place between proteins, lipids and variable cofactors, artificial membranes are very useful. The PsbH protein is associated with the reaction centre of PSII in higher plants, algae and cyanobacteria. In our study *psbH* gene from cyanobacterium *Synechocystis sp.* PCC 6803 was cloned into a plasmid expression vector, which allowed a synthesis of the PsbH protein as a glutathione-S transferase (GST) fusion protein in *E. coli* BL21(DE3) cells. Although the exact role of the protein PsbH is not clear, it seems to be important for the structure and function of photosystem II. These structural and functional role could be closely associated with lipidic environment surrounding the protein. Moreover the protein could bind some cofactors e.g. pigments or in literature mentioned carbon dioxide [1].

Lipids were extracted from Synechocystis sp. PCC 6803 using method of Bligh and Dyer [2]. Extracted lipids were used to prepare liposomes by reversed phase evaporation. The detergent mediated reconstitution was performed according to Lévy et al. [3]. Interaction of lipids and other bound compounds was monitored by measurement of circual dichroism. Interaction of chlorophyls and protein was detected by low temperature fluorescence.

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STRUCTURAL BIOLOGY ON THE SODIUM PUMP: A COMBINED APPROACH LEADING TO A FULL CHARACTERIZATION OF THE CATALYTIC DOMAIN

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In this paper we summarize our previous work on the catalytic part of Na^+/K^+-ATPase. The nucleotide-binding domain of the subunit of mouse brain of Na^+/K^+-ATPase was expressed and isolated from *Escherichia coli* cells. The secondary structure of the expressed domain was experimentally determined by UV circular dichroism and Raman spectroscopy. By computer modeling was generated a three-dimensional model with and without docked ATP and predicted amino acids involved in the ATP binding site. ATP binding of wild type was followed by Raman difference spectroscopy and point mutants were measured by fluorescence spectroscopy with TNP-ATP. The set of eight amino acids residues was identified to form the complete ATP recognition site.

for full paper see page 18

APPLICATION OF DEGENERATE OLIGONUCLEOTIDE GENE SHUFFLING FOR CONSTRUCTION OF HYBRID HALOALKANE DEHALOGENASES

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Haloalkane dehalogenases are bacterial enzymes catalyzing cleavage of the carbon-halogen bond of halogenated aliphatic compounds by a hydrolytic mechanism. Improvement of catalytic properties of these environmentally important enzymes can be reached by application of non-recombinant directed evolution techniqes [1,2] or recombining several homologous genes [3].

Four haloalkane dehalogenase genes of different origin were used to construct a hybrid gene: *dhlA* cloned from *Xanthobacter autotrophicus* GJ10 [4], linB from *Sphingomonas paucimobilis* UT26 [5], *dhaA* from *Rhodococcus rhodochrous* NCIMB13064 [6] and *dhmA* from *Mycobacterium avium* N85 [7]. The technique called Degenerate Oligonucleotide Gene Shuffling was used for *in vitro* recombination of four different genes [8]. Altogether twelve hybrid genes were constructed using one pair of degenerate oligonucleotides.

For preliminary characterization, hybrid proteins were expressed in *Escherichia coli* BL21(DE3) and tested in the resting cells assay for activity towards six halogenated aliphatic compounds. Four out of twelve hybrid proteins keep good expression and ten proteins showed obvious catalytic activity. Comparison of relative activities determined for the hybrid enzymes with the activities of wild type enzymes suggests that constructs do not possess novel substrate specificities.

All hybrid genes were cloned to pET-32(a) vector to support high level of expression an stability of hybrid proteins. His-taggged tail was introduced to C-terminus of hybrid proteins. All hybrid haloalkane dehalogenases were successfuy expressed in fusion with thioredoxin in host cells *E. coli* HB101. Optimization of purification conditions on Ni-NTA agarose and cleavage of hybrid protein-thioredoxin complexes is under progress.

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TOWARDS TRUE SSTABILIZATION ENERGIES OF H-BONDED AND STACKED DNA BASE PAIRS

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The study of binding energies of Adenine...Thymine and Guanine...Cytosine base pairs in vacuo was aimed to get an information on relative order of different structure motives on the energetical scale with special focuse on differences between H-bonded and stacked structures. We also aimed to find a general way to reliable interaction energies of the weakly bonded complexes.

Structures taken from previous MD/quench studies were fully optimized at RI-MP2 [1,2] level with TZVPP [5s3p2d1f]/[3s2p1d] basis set. To approach complete basis set (CBS) limit, convergency of both HF energy and MP2 correlation energy was studied employing augmented correlation-consistent basis sets aug-cc-pV(D,T,Q)Z. Is was found that molecular interaction energies close to the CBS limit may be obtained by 2-point extrapolation [3] using aug-cc-pVDZ and aug-cc-pVTZ basis sets.

To account for higher order correlation effects convergency of CCSD(T) MP2 correction term (E_{corr}^{MP2} - E_{corr}CCSD(T)) was investigated. For the formamide… .formamidine complex (Fig. 1) which is a model for adenine..thymine interaction MP2 and CCSD(T) correlation

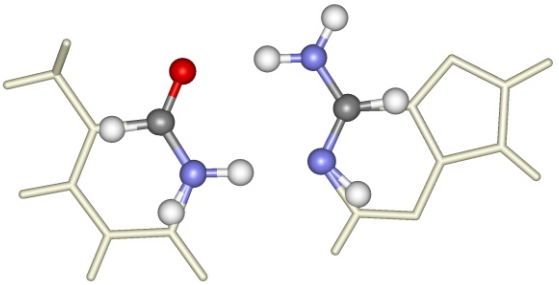

Figure 1

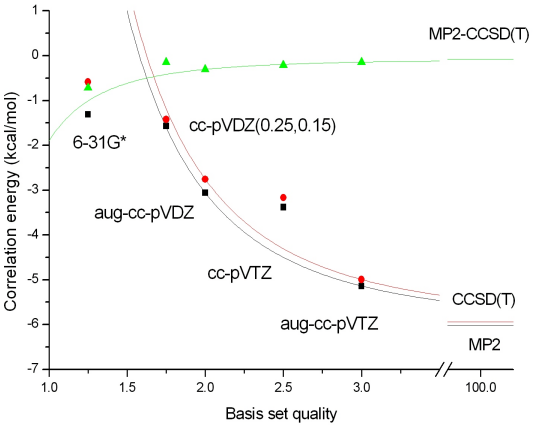

Figure 2

interaction energies were evaluated with various basis sets up to aug-cc-pVTZ (Fig. 2). It was shown that unlike the correlation energy itself the CCSD(T) MP2 difference is almost basis set independent. Rather accurate values were obtained with relatively small 6-31G*(0.25) and cc-pVDZ(0.25,0.15) basis sets. Because the latter one performs well also for stacked complexes [4] it can be recommended for evaluation of the term of extended complexes possessing both H-bonded and stacked structures.

Interaction energies of the DNA base pairs obtained by combination of the CBS extrapolations of MP2 interaction energies and the CCSD(T) MP2 correction are in good agreement with experiment.

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POTENTIAL ENERGY SURFACES OF GUANINE - CYTOSINE BASE PAIR AND RELATED TAUTOMERS: MOLECULAR DYNAMICS AND AB INITIO STUDY

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Introduction:

The structure of DNA is determined among other factors by interactions between nucleic acid (NA) bases: guanine (G) , cytosine (C), adenine (A) and thymine (T). A theoretical study of the interaction is important for understanding of stabilizing forces in DNA and RNA. The interaction of NA bases in a vacuum in now being studied in experimental laboratories [1-4] and a knowledge of the potential energy surfaces is essential for an interpretation of experimental results. This information can be obtained by performing correlated *ab initio* calculations in combination with molecular dynamics/quenching technique (MD/Q) [5-6].

Methods:

1. *Molecular dynamics/ quenching calculations* were carried out in the NVE canonical ensemble (Constant number of particles, volume and energy) employing Cornell *et al.* AMBER force field [7], which gives results comparable with *ab initio* data [8]. Due to comparable stability of several cytosine and also guanine tautomers, all possible combinations of these tautomers should be considered. Only the most stable (stabilization energy higher than 18 kcal/mol) and populated (population greater than 5%) structures of base pairs were taken for further *ab initio* calculations.

2. *Ab initio calculations*. The geometries, interaction and tautomerization energies of base pairs were determined on RI-MP2 level employing TZVPP (5s3p2d1f/ 3s2p1d) basis set.

Results:

In all cases planar H-bonded structures are the most stable and most populated ones. The T-shaped and stacked structures are about several kcal/mol less stable (typically 5-10 kcal/mol) than the structure of the global minimum and will not be probably detectable by experimental technique.

Among all possible combinations of tautomers the highest stability shows canonical Watson-Crick (WC) structure (-26.9 kcal/mol) followed by the same binding pattern with N7 keto tautomer of guanine Also other binding patterns of ketoguanine-ketocytosine tautomers are very stable. The structures of other combinations of tautomers are usually less stable (about 4-5 kcal/mol) than the WC pair, including ketoguanine-enolcytosine structure observed in the experiment [2]. An exception is an enolguanine - ketocytosine nonplanar structure with surprisingly high stability (-25.3 kcal/mol), but due to unfavorable geometry and stability of the enolguanine tautomer itself, this structure will not be probably detectable.

Summary:

We have presented a powerful technique for scanning of potential energy surfaces of nucleic acid base pairs, which can be used for analysis of experimental results. It is demonstrated that the use of standard procedure based on chemical feeling and experience is not sufficient and several mainly unusual structures can be omitted.

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THE ROLE OF INTER-HELICAL INTERACTIONS IN ELECTRON-TRANSFER GATING IN PHOTOSYSTEM II

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Non-covalent interactions between transmembrane (TM) helices of membrane-protein complexes may affect subunit association and domain flexibility. Conformational changes of flexible protein domains have been suggested to be involved in electron-transfer gating in photosynthetic reaction centers (RC). We set out to study the role of non-covalent inter-helical interactions between two subunits of photosystem II (PSII) in the gating mechanism of electron-transfer between the quinones QA and QB.

The core of PSII RC is made of two protein subunits, D1 and D2. Two TM helices of these subunits are associated in their membranal region through a single hydrogen bond (H-bond) embedded within a common helix packing motif (GxxxSxxxG). A putative H-donor in this contact point, D1-Ser212, was mutated to all other amino acids in the cyanobacterium Synechocystis sp. PCC 6803.

Thirteen mutations were found to support photoautotrophic growth excluding bulky residues that are positively charged (Arg, Lys), or aromatic (Phe, Trp, Tyr and His). In the photoautotrophic mutants, the effect of the mutations on forward electron transfer rates and charge recombination was evaluated following fluorescence decay and thermoluminescence as a function of temperature. The results showed that weakly polar residues such as Ser (wild type), Thr, Ala and Cys had similar rate constants (kAB) of electron transfer over a range of temperatures (10 – 40 °C). On the other hand, strong polar and/or bulkier residues, such as Gln, Asn, Glu and Asp, had lower kAB, which increased in a temperature-dependent manner. The temperature effect on kAB varied among the mutants suggesting that protein conformations influence electron transfer rates. Moreover, good correlations were found between the activation enthalpies of forward electron transfer dH‡ and the activation energy of charge recombination (EA), and between dH‡ and mean packing values, especially when comparing residues that can form H-bonds. These findings suggest that weak hydrogen bonding and polar interactions at the TM helical interface between the D1 and D2 subunits affect local protein conformations involved in the gating of electron-transfer from QA to QB in PSII.

NATURE OF BINDING OF BOMBYKOL IN PHEROMONE BINDING PROTEIN. AN AB INITIO STUDY

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Interactions of insects with their surroundings are mostly based on chemical signals. One of the most remarkable communication systems known mediates sexual behavior of moths. Mature females ready to have offspring emit a sexual pheromone from their abdomen to attract conspecific males for mating. The 'single-pheromone molecule' tuned detection system of males is located in branches of male's antennae. On these antennae olfactory hairs, *sensila trichodea*, are located. They are filled with sensilar liquor and house specialized dendritic cells, innervated to insect brain globular structures. Here, the signal received from the cell is proceeded and further recognized as a call for copulation.

The sensilar liquor contains a high concentration (10 mM) of water-soluble pheromone-binding-protein (PBP).

An analysis of the crystal structure of Bombyx mori PBP···bombykol (pheromone) complex [1] identified nine amino acid residues involved in intermolecular hydrogen bonds, ··· interactions, C-H·· hydrogen bonds and weak interactions of purely van der Waals character. Using the model fragments as the representatives of each residue, the interaction energies of their complexes with bombykol were computed by *ab initio* calculations. The values were compared with literature and further discussed in terms of the method and basis set dependence, and the co-operative effect (influence of the neighboring groups on the interacting pair). It enabled us to explain quantitatively the nature of the binding forces in [BmPBP···bombykol] complex in terms of contribution of the individual amino acids and individual types of interaction. It was observed that 70% of the stabilization is due to interactions other than classical hydrogen bonds.

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MODELLING OF ENZYME-SUBSTRATE COMPLEXES FOR COMBINE ANALYSIS OF HALOALKANE DEHALOGENASE BY MEANS OF MOLECULAR DOCKING AND QUANTUM MECHANICAL CALCULATIONS

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The applicability of automated molecular docking techniques and quantum mechanical calculations for the construction of enzyme-substrate complexes for use in Comparative binding energy (COMBINE) analysis [1-6] was evaluated. The data set studied consists of the complexes of eighteen substrates with the haloalkane dehalogenase (DhlA) isolated from bacterium *Xanthobacter autotrophicus* strain GJ10. An automated molecular docking procedure provided the structures for a set of DhlA-substrate complexes that was used to derive a robust COMBINE model. Quantum-mechanical calculations were successfully used as an additional and complementary computational tool for selection of correct binding modes obtained from the docking. The resulting COMBINE model is compared with a previously reported COMBINE model [7] derived for the same data set using structures of complexes built according to experimentally determined structure of the DhlA-dichloroethane complex. Both models were similar in terms of overall fit and internal predictive power even though the conformations and orientations of the substrates in the complexes were significantly different. The new COMBINE model derived from the automatically docked structures performed notably better in external prediction. Small differences in the relative contributions of important residues to explaining binding affinities can be directly linked to structural differences in the modelled enzyme-substrate complexes.

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SECONDARY AND TERTIARY STRUCTURE OF HUMAN $_1$-ACID GLYCOPROTEIN BY HOMOLOGY MODELING AND VIBRATIONAL SPECTROSCOPY

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Human $_1$-acid glycoprotein (AGP), also known as orosomucoid, is a 41-kDa single polypeptide formed of 183 amino acids. It contains 42% carbohydrate in weight and has up to 16 sialic acids residues. AGP, a human blood plasma protein, belongs to the lipocalin family of proteins, a heterogeneous group of proteins that bind a variety of small hydrophobic ligands. It is known that AGP plays a role under inflammatory or other pathophysiological conditions and is able to bind basic drugs and certain steroid hormones such as progesterone, however its biological function and 3D structure remains unknown [1].

The aim of our work was to predict and verify the three-dimensional structure of AGP. A structural model, using available lipocalin structures as templates, was constructed by means of the Modeller program [2]. The model shows that AGP folds as a highly symmetrical all- protein dominated by a single eight-stranded antiparallel -sheet. For the first time secondary and tertiary structures of AGP have been studied by infrared and Raman spectroscopy. Vibrational spectroscopy confirmed details of the secondary structure predicted by modeling, i.e. 15% -helices, 41% -sheets, 12% -turns, 8% -bands and 24% unordered structure at pH 7.4. Thermal dynamics in the range 20-70 °C monitored by Raman spectroscopy and analyzed by principle component analysis revealed full reversibility of the protein motion upon heating dominated by decreasing of -sheets, probably thermal "breathing" of the -barrel.

Docking of progesterone into the binding pocket of our model was explored with the AutoDock program [3]. Then Raman difference spectroscopy confirmed the predicted

proximity of Trp122 to the progesterone binding pocket. We can conclude that our model was verified in so many details by vibrational spectroscopy that it can represent a valuable contribution to understanding the role and behavior of AGP [4].

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MOLECULAR CLONING, E. COLI EXPRESSION AND PURIFICATION OF SCFV ANTIBODY FRAGMENTS OF DIAGNOSTIC/THERAPEUTIC INTEREST

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Fv fragments are the smallest antibody molecules that still retain the entire antigen-binding site. In single-chain Fv fragments (scFv), variable domains are joined by a flexible linker. Such scFv constructs, which generally retain full binding capacities towards the antigen, are the topic of very active research. First, they are of interest for structural studies because they usually yield crystals diffracting to higher resolution than the corresponding Fab fragments. Second, since they can be expressed in bacteria, they are also well suited for binding, mutagenesis and protein engineering studies [for review, see e.g.1].

In this work we describe molecular cloning, expression, purification and properties of two scFvs of potential diagnostic and immunotherapeutic use, scFv M75 and scFv TU?20.

Monoclonal antibody (mAb) M75 recognizes cell surface protein MN/CA IX strongly associated with several types of human carcinomas [2]. Radioactively labeled humanized scFv M75 could be used for tumor immunodetection and possibly for therapy. Monoclonal antibody TU-20 was raised against beta-III-tubulin, a specific neuronal marker in normal and neoplastic tissues. The antibody TU-20 fragments could thus be useful tools for probing beta-III-tubulin functions in neurons, as well as for immunohistochemical characterization of tumors of neuronal origin [3].

Coding sequences for light (V_L) and heavy (V_H) variable domains were obtained from total RNA, isolated from hybridoma cells, by RT-PCR using suitable pairs of primers. Single-chain Fv genes in the form V_L-linker-V_H-myc tag were then assembled and cloned into T7 promoter-driven expression plasmids. Bacterial strain *E. coli* BL21(DE3) was used for protein expression. The recombinant protein products accumulated in inclusion bodies as insoluble aggregates. To obtain refolded active proteins from inclusion bodies, several protocols were adapted to find optimal conditions for each scFv species.

Purification procedure comprising several conventional chromatography steps (ionex chromatography, gel filtration) yielded scFv proteins in amount and purity necessary for functional characterization. While scFv TU-20 in ELISA assay exhibits specificity and binding activity comparable to parental mAb TU-20, in case of scFv M75, ELISA assay was negative. The reasons for the lack of binding activity are under investigation.

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PROTEINS AND THEIR CRYSTALS

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Non-membrane proteins such as the pokeweed antiviral protein from Phytolacca acinosa (PAP-Saci) and the tryptophan (W)-repressor binding protein A (WrbA) and also membrane protein, the five-chlorophyll reaction center of photosystem II from Pisum sativum, have been crystallized in our laboratory.

for full paper see page 30

NANOSECOND MOLECULAR DYNAMICS OF HIV PROTEASE- INHIBITOR COMPLEXES:INSIGHTS INTO THE DIFFERENTIAL BINDING POTENCY OF DIASTEREOISOMERES

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The inhibitory potency of four nanomolar diastereomeric inhibitors of HIV-1 protease [1] was studied by molecular dynamics simulations and MM-GBSA/PBSA analysis. As a starting point we used the crystal structures of protease-inhibitor complexes [2, 3]. Having added hydrogens, we surrounded the complexes with a box of explicit water molecules and added counterions to neutralize the box. Using AMBER 7 program package [4], we minimized, heated and equilibrated the system after which we ran 2-nanosecond-long production dynamics. Periodic boundary conditions were used and long-range electrostatics was treated by particle mesh Ewald (PME) technique.

An analysis of the molecular dynamical trajectories was performed and their quality assessed. The protease-inhibitor binding energies were calculated with MM-GBSA/PBSA approach. The effect of the length of the simulation, method to calculate solvation energy, and other factors upon the results was determined.

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FULL CHARACTERIZATION OF NATURAL KILLER CELL MEMBRANE MICRODOMAINS

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Natural killer (NK) cells are cytotoxic effector lymphocytes, which do not express antigen-specific cell surface receptors. NK receptors that mediate signals leading to the initiation or supression of natural cytotoxicity processes are poorly characterized. Moreover, these receptors may associate with other co-stimulating molecules and adaptor proteins, which transduce the signal from the receptor to the cell. Such complexes may be observed as biochemically distinct parts of plasmatic membranes and are commonly referred to micro domains or glycosphingolipid enriched microdomains (GEMs). They are enriched in GPI-anchored and acetylated proteins and in cholesterol and glycosphingolipids. It is believed that they may aid in signal transduction as well as in trafficking through the secretory and endocytic pathways and in cell to cell interaction.

In this study we focused on membrane microdomains from rat NK leukaemia cell line (RNK-16). For detailed characterization we used a shotgun strategy based on microcapillary HPLC - tandem mass spectrometry. Aditionally, we applied techniques of native electrophoresis for detailed mapping of protein complexes present in the GEMs. We have identified a large number of proteins (e.g. gp-42, CD2, LAT, CD161, CD44 or g-proteins in GEM and tubuline in non-GEM fractions).

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HIGH AFFINITY LIGANDS FOR HUMAN LYMPHOCYTE RECEPTOR CD69

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CD69 is one of very important activating receptors expressed on the surface of human lymphocytes. This molecule exists as a homodimer, each of its subunits being terminated with the globular domain related to the C-type lectin family. Although the crystal structure of this domain has been recently solved [1], its potential ligands and the function of the whole receptor remain unclear. In our labo-

ratory [2] we established that calcium ion can be bound tigtly in the molecule and this binding increases the affinity of the protein to N-acetylglucosamine and N-acetylgalactosamine. The positions of binding sites has been suggested by molecular modeling and proved by site-directed mutagenesis.

These data allowed us to find potential high affinity ligands among branched oligosaccharides terminated with N-acetylglucosamine units. We isolated these molecules by deglycosylation of ovomucoid and characterized them by mass spectrometry. From results of our binding studies we can conclude that pentaantenary structure is the ligand with the highest known affinity for CD69 molecule. It has been published [3, 4] that similar structures are expressed on the surface of some tumor cells. This finding indicates that one role of CD69 molecule on the cells of the immune system may be to attract killer lymphocytes to the tumor sites.

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A DFT INVESTIGATION OF STRUCTURE-CHEMICAL SHIFT RELATIONSHIPS FOR 13C AND 15N IN DNA

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Density functional theory has been applied to explore the dependence of ^{13}C and ^{15}N chemical shifts in deoxyribonucleosides on various structural features such as the orientation about the glycosidic bond, the CH2OH group conformation, the sugar pucker, and the hydrogen bonding. Geometry optimizations have been performed with sugar-phosphate backbone dihedral angles frozen to their average experimental values in BI-DNA. Results obtained in NMR parameter calculations have been compared to available experimental data for C1`, C2` and N9.

The effect of the glycosidic torsion angle has already been studied [1] but we wished to involve the relaxation of the geometry after changing , which has not been considered in the previous work [1]. C1`, C2` and N1/N9 chemical shifts appeared to be influenced most by the base orientation. The trends uncovered in chemical shifts are significantly different from those reported previously [1] and the absolute chemical shift values are in the case of C2` approximately the same for all deoxyribonucleosides, except for the anti orientation of the base. On the contrary, for C1` and N1/N9 the trends for purine nucleosides differ from those for pyrimidine nucleosides and the absolute N1 chemical shifts in deoxycytidine are found upfield relative to deoxythymidine.

Besides the influence of varying the glycosidic torsion angle, we wanted to assess the effect of the sugar puckering and the hydroxymethyl rotation, both of which were studied on deoxyguanosine. N9 experienced the largest changes, namely 10 or 8 ppm difference between the south and north conformation in both the syn and anti region, respectively. The N9 chemical shift for deoxyguanosine (*S, anti, gg*) differed significantly from the other two CH_2OH-rotamers.

The comparison with the experiment has been carried out using the data from BMRB database [2] (C1`, C2`) and the data for the $[d(G_4T_4G_4)]_2$ quadruplex (C1`, N9) [3], on which changes upon the hydrogen bonding have also been studied.

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NATURE OF STACKING INTERACTIONS BETWEEN INTERCALATORS AND DNA BASE PAIRS. AB INITIO QUANTUM-CHEMICAL, DENSITY FUNCTIONAL THEORY AND EMPIRICAL POTENTIAL STUDY.

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Properties of isolated intercalators (ethidium (E), daunomycin (D), ellipticine (EL) and 4,6'-diaminido-2-phenylidone (DAPI)) and their stacking interactions with adenine…thymine (AT) and guanine…cytosine (GC) nucleic acid base pairs were investigated by means of a nonempirical correlated *ab initio* method [1]. All intercalators exhibit large charge delocalization and neither of them (including dicationic DAPI) exhibit a site with dominant charge. All intercalators have large polarizability and are good electron acceptors while base pairs are good electron donors. MP2/6-31G*(0.25) stabilization energies of complexes intercalator…base pair are large (E…AT : 22.4 kcal/mol; D…GC :17.8 kcal/mol; EL…GC :18.2 kcal/mol; DAPI…GC :21.1 kcal/mol) and are well reproduced by modified AMBER potential (vdW radii of intercalator atoms are enlarged and their vdW energy depths are increased). Standard AMBER potential give less satisfactory results especially for DAPI containing complexes. Because DAPI is the best electron acceptor (among all

intercalators studied) this difference is explained by the importance of the charge transfer term which is not included in the AMBER potential. The Hartree-Fock and DFT/B3LYP methods not covering the dispersion energy fail completely to describe any energy minimum at the potential energy curve of the E…AT complex and these methods thus cannot be recommended for a study of intercalation process. On the other hand, a modified version of DFT method which covers London dispersion energy yields for all complexes very good stabilization energies well comparable with referenced *ab initio* data. Besides vertical dependence of interaction energy twist dependence of interaction energy was also investigated by both, reference correlated *ab initio* method as well as empirical potentials. It is concluded that despite the charged (E +1, D +1, DAPI +2) or polar (EL) character of intercalators investigated it is the dispersion energy which predominantly contributes to the stability of intercalator…DNA base pair complexes. Any procedure which does not cover dispersion energy is thus not suitable for studying the process of intercalation.

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ANALYSIS OF INTERACTIONS IN COMPLEXES OF HIV-1 PROTEASE AND ITS PEPTIDOMIMETIC INHIBITOR

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HIV-1 protease is a 22 kDa protein of the human immunodeficiency virus. The function of this protein is to cleave polyprotein of immature virus and thus to contribute to formation of active matured virus. Inhibition of the protease is therefore one of possible ways of fighting with disease AIDS, caused by the human immunodeficiency virus.

Our research was focused on interaction analysis of HIV-1 protease and its peptidomimetic inhibitor Boc-Phe- $[CH_2CH_2NH]$-Phe-Glu-Phe-NH_2, denoted as OE. The inhibitor was developed in the laboratory of J. Konvalinka (Institute of Organic Chemistry and Biochemistry, Academy of Sciences CR). Native and mutant (A71V, V82T, I84V) HIV-1 protease were expressed and purified in the laboratories of J. Sedláček (Institute of Molecular Genetics, Academy of Sciences CR) and J. Konvalinka. In our research group, crystallization of complexes of OE with native and mutant protease was performed, X-ray diffraction of crystals on the synchrotron source of radiation was measured and structures of both complexes were determined ([1], [2]).

As a result, we have two structures with *R*-factors 18 % (native protease complex, diffraction limit 2.45 Å) and 20.3 % (mutant protease complex, diffraction limit 2.2 Å). Both complexes crystallized in space group P61 and inhibitor OE was found in the active site in two approximately C_2 symmetrical positions, following thus pseudosymmetry of the protease. This fact makes interpretation of interactions between the protease and inhibitor more difficult. Therefore, standard structural analysis of contacts between the protease and inhibitor was completed by two energy analyses of interactions in the active site. The inhibitor binding modes to both proteases are similar from the structural point of view and interpretation of small details could be ambiguous. However, energy analysis of both complexes confirms the interpretation of changes caused by mutation of the protease. Mutated residue Thr 182 forms an aromatic hydrogen bond to the inhibitor phenyl group in P1 position. Mutation I84V causes a decrease in van der Waals interaction between residue 84 and the OE inhibitor.

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MEMBRANE PSEUDO-CRYSTAL STRUCTURES IN PSSU-IPT TOBACCO CHLOROPLASTS

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Our study is focused on native pseudo-crystalline structures, which were observed in chloroplasts of transgenic tobacco overproducing plant hormones cytokinins. The structures were not positively identified until now. We suppose that they are formed by light harvesting protein (LHC) aggregating in a form of 2D crystal, which then constitute membrane stacks. Our hypothesis is supported by fluorescence emission spectra, which showed certain bands corresponding to LHC aggregates and higher emission of chlorophyll *b* in chloroplasts izolated from transgenic plants.

The aim of this experiment was the estimation of relative size of pseudo-crystals compared to chloroplast and the size of basic cell unit, which can be determined from analysis of TEM images from ultrathin sections of leaves and isolated chloroplast suspensions.

Transgenic tobacco containing a supplementary iptene under a control of the promoter for the small subunit of

RuBPCO (P*ssu-ipt*) was grown as grafts on non-transgenic tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) rootstock as described by Synková et al. (J. Plant Physiol. 155: 173-182, 1999) or as rooted plants (kanamycin resistant progeny of the transgenic grafts). Samples for TEM were taken from the central part of the young fully developed leaf or isolated chloroplast suspension and after overnight fixation in 3 % glutaraldehyde were embedded in Spurr's resin. Ultrathin sections were stained by uranyl acetate and Reynold's lead citrate and examined in JEM 1010 (Jeol, Japan). Analysis of serial sections by program IMOD 2.42 enabled three dimensional (3D) reconstructions of chloroplasts and pseudo-crystals. The size of basic structural unit was calculated using MRC Cambridge Image Processing System (1994).

3D reconstruction showed that pseudo-crystalline structures occupy up to 20 % of chloroplast volume (at least in that part of chloroplast which was studied).

The average size of basic cell unit was calculated as: a = 11 nm, b = 12 nm, = 100 .This size parameters support our hypothesis, although dehydration preceding embedding in epoxide resin cause a shrinkage of natural structures. Therefore further experiments are needed to prove it.

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THE ROLE OF STRUCTURAL DIFFERENCES OF FLAVANOLIGNAN SILYBIN STEREOIZOMERS IN BINDING TO HEPATOCYTES

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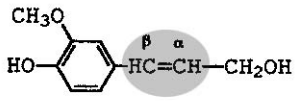
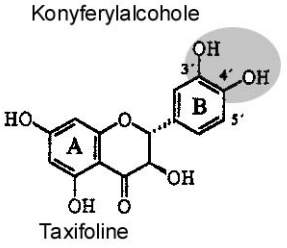
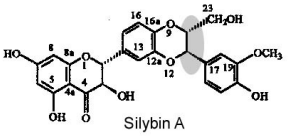
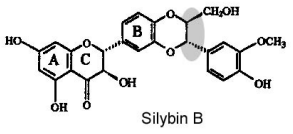
Hepatoprotective effects of Milk Thistle (*Silybum marianum*) have been known since ancient Greece and Roma very well. Flavanolignans (called silymarine) extracted from Milk Thistle seeds were shown to help against hepatotoxic effects of many natural toxins (i.e. alga toxin microcystine, mushroom toxins amanitin and phaloidin, fungal toxins cyclosporines, etc.). The main active substance of silymarin is silybin.

Fig. 1









Recent studies revealed that many transport and metabolic processes in the cell are stereospecific. Silybin occurs in two stereoisomers (A and B) that differ in the bound between konyferyl and taxifolin (Fig. 1). We developed a new method for preparation and purification of these silybin stereoisomers and for their specific labelling by radioactivity (3H, 125I) at positions 6 and 8. Transport of four stereoisomers was studied. The best affinity of transport systems were found for 6-[125I]silybinA, which is taken 100 times better than the other silybin stereoisomers.

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A THEORETICAL STUDY OF THE PHOTOSYNTHETIC REACTION CENTRE PSII

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Mutlireference perturbation theory (CAS-PT2), random phase approximation (RPA), configuration interaction with singles (CIS) (all at various basis sets) and semiempirical ZINDO methods were used for the determination of the excited states energies of free-base porphin and Mg-porphin. On the basis of these results an error estimation of the methods used for larger systems (molecules in PSII reaction center) calculation can be established.

The PSII model in ref. (1) was used for the electron-exication spectra determination. First, individual ZINDO spectra of monomers chlorophyll-a and pheophytine-a were estimated for semiempirically (PM3) fully optimized structures.

As a next step, partial optimizations (the hydrogen atoms only) on all the monomers, selected dimers, trimers, tetramers, and hexamer were done at the same PM3 semiempirical level, keeping the positions of all the "x-ray determined atoms" fixed. Selection of studied dimeric and

oligomeric structures is based on the considerations of the multiparticle (multimolecular) excitations. These multiparticle specta exhibits complex non-additive character where all the basic spectra lines (Qx, Qy, Soret lines) are shifted unevenly from their positions in isolated molecules. This uneven character speaks out about the different importance of individual molecules in the various excitations.

Another comparison of electron-excitation spectra using ZINDO and RPA was performed for the chlorophyll-a and pheophytine-a molecules, as well. For deeper elucidation, the structures used in comparison were both fully-optimized and "hydrogens-optimized" (taken from RC model).

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OVEREXPRESSION AND PURIFICATION OF RECOMBINANT MEMBRANE PROTEIN PSBH IN ESCHERICHIA COLI

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In this work we featured an expression system that enables the production of sufficient quantities of membrane PsbH protein (~mg´s quantities) for solid-state NMR as well as other biophysical studies. PsbH is a small membrane protein associated with the photosystem II complex in higher plants, algae and cyanobacteria. Although the exact role of PsbH is not clear, it seems to be important for the structure and function of photosystem II.

In this approach a synthetic psbH gene from cyanobacterium *Synechocystis sp.* PCC 6803 was cloned into a plasmid expression vector, which allowed a direct synthesis of the PsbH protein as a glutathione-S transferase (GST) fusion protein in *E. coli* BL21(DE3) cells. A relatively large GST anchor overcome foreseeable problems with the low solubility of membrane proteins and the toxicity caused by protein incorporation into the membrane of the host organism. As a result, the majority of fusion protein was obtained in a soluble state and could be purified from crude bacterial lysate by affinity chromatography on immobilised glutathione under non-denaturating conditions. The PsbH protein was cleaved from the carrier protein with Factor Xa protease and purified on DEAE- cellulose column with yields of up to 2.1 μg protein/ml of bacterial culture. Details of sample optimization for small membrane proteins as well as the impact constitutive cell protection mechanism against host membrane proteins are discussed.

KINETIC AND STRUCTURAL CHARACTERIZATION OF TWO ACTIVE FORMS OF ASPARTIC PROTEINASE FROM MURINE INTRACISTERNAL A-TYPE PARTICLES

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Murine intracisternal type A particles (intracisternal A-particles, IAPs) are endogenous retroviruses encoded by many proviral elements within the mouse genome. They share sequence homology with the B-type mouse mammary tumor virus (MMTV), the D-type simian retroviruses (SRV) and C-type avian sarcoma virus. IAPs genetic elements have been shown to transpose within the genome of retrovirus-producing cells.

The IAP particles assemble and bud at the membranes of the endoplasmic reticulum (ER) where they accumulate as immature particles consisting exclusively of uncleaved polyproteins. They do not leave cell and horizontal transmission via free particles has not been achieved. Recent evidence has indicated that the lack of proteolytic processing is not due to a defective viral proteinase but rather is caused by the site of particle formation and can be rescued by an artificial redirection of the polyprotein to the plasmatic membrane.

The recombinant proteinase of murine intracisternal A-type particle 14 (MIA14 PR) undergoes N- and C- terminal autoprocessing at defined sites and is sequentially and functionally related to the B- and D-type retrovirus proteinases. An unusual feature of these proteinases as opposed to the C-type retrovirus proteinases is a 50 amino acid C-terminal extension of unknown function. In this study, we aim to analyse the possible role of the C-terminal extension of the proteinase in regulation of polyprotein processing.

We have cloned, expressed in *E. coli* and purified to homogeneity both the full-length MIA14 PR and its C-terminally truncated form. Both enzymes are active and have been used for *in vitro* kinetic studies using peptide substrates and inhibitors. The C-terminal extension of MIA14 PR has been cloned, expressed in *E. coli*, purified and its effect on catalytic activity of both MIA14 PR constructs evaluated.

THE VITAMIN B12 BIOSYNTHETIC PATHWAY: STRUCTURE ANALYSIS OF UROPORPHYRINOGEN-III C-METHYL-TRANSFERASE

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The biosynthesis of vitamin B_{12}, "the anti-pernicious anaemia factor", requires about 30 enzymes, and is further complicated by the appearance in nature of two separate pathways, representing aerobic and anaerobic routes, where the major difference seem to be concerned with the process of cobalamin ring contraction and cobalt chelatation. *Pseudomonas aeruginosa* is able to synthesise the vitamin in the absence of oxygen. However, the bacterium can also make B_{12} when grown aerobically. Thus, there must exist a pathway that can operate both in the presence and absence of molecular oxygen.

Uroporphyrinogen (uro'gen) III methyltransferase, a key enzyme in the biosynthetic pathways of vitamin B12 and siroheme, catalyzes the S-adenosyl-L-methionine (SAM)- dependent bismethylation of its substrate, uro'gen III, resulting in the formation of dihydrosirohydrochlorin (precorrin-2). The enzyme exists in at least two forms. One form, encoded by the *cob*A gene, is required for vitamin B_{12} synthesis in *Pseudomonas denitrificans*. The second form, encoded by the *cys*G gene, is required for siroheme in *E. coli*. Both forms of the enzyme perform the *in vivo* synthesis of precorrin-2, but in addition, CysG has NAD+-dependent precorrin-2 oxidase and ferrochelatase activities. The CysG enzyme mass is ~52 kDa, whereas the smaller CobA protein mass is of ~30 kDa and is homologous only to the C-terminal region of CysG.

CobA is a key regulatory enzyme in the branched tetrapyrrole biosynthetic pathway, and is sensitive to both substrate and product inhibition. To gain some molecular insight into how this enzyme exerts its control, we have crystallised the CobA protein and collected data on the SRS synchrotron in Daresbury. The molecular replacement method (AMoRe) has been used for the phase problem solution with the C-terminal domain of CysG as a search model. Structure refinement is currently under way.

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INTERACTION CISDDP WITH BASES OF DNA

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In previous study [1], two-bases (adenine-adenine, adenine-guanine and guanine-guanine) interactions with cisplatine were examined using ab initio methods. Current study represents further extention of that work including sugar-phosphate backbone which connects bases. Starting geometries of the studied systems (ApA, ApG and GpG) were based on X-ray Pt-GpG structure[2]. The optimizations were performed using density functional Becke3LYP with 6-31G* basis; for platinum and phosphor pseudopotential description was used. Single-point second order Moller-Plesset perturbation theory (MP2) was used for the DFT-optimized structures. Then sugar-phosphate chain was removed and B-Pt-B bridged systems (B=A and G) were calculated (also at MP2/6-31G* level). Using these calculations, bond dissociation energies (BDE) of two bonds between Pt and N_7 site at purine were determined for systems with and without sugar-phosphate string. Systems without sugar-phosphate string were also calculated at MP2/6-31+G* level, and BDE were determined for each Pt-B and Pt-NH_3. These data were compared with energies from study [1].

It was shown that close correspondence can be found between Pt-N_7 BDE's for systems optimized without ([1]) and with (this work) sugar-phosphate backbone when these backbones are not considered. Similar comparison can be done within current model where the role of sugar-phosphate string can be elucidated. Analogous Pt-N_7 BDE are additionally influenced mainly with coulomb interaction between negatively charged phosphate group and Pt cation. This causes an increase in BDE up to 40 kcal/mol in Pt-ApA complex.

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