# 1st MEETING OF THE CZECH AND SLOVAK STRUCTURAL BIOLOGISTS

The conference took place in the Academic and University Center in Nové Hrady on March 14 - 16, 2002. Scientific programme was prepared by Dr. Bohdan Schneider, local organisers were Dr. Kutá Smatanová, Ing. Strouhová and Dr. Štys.

Abstracts of some of the contributions presented at the conference as lectures or posters can be found in this section of Materials Structure. A short report on the conference and announcement of the 2nd meeting will be published in next issue of the journal.

## DIFFERENTIATION AND ASYMMETRIC CELL DIVISION IN *Bacillus subtilis*: GENETIC AND STRUCTURAL STUDIES OF PROTEINS INVOLVED

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Eukaryotic and prokaryotic cell division requires accurate spatial co-ordination. A fundamental problem in cell division concerns the mechanisms that ensure correct placement of the septum. In *Escherichia coli* and *Bacillus subtilis*, the first step in the assembly of the cell division apparatus is the localisation of an FtsZ ring at the site of septation. While, the Min system blocks polar septation in *B. subtilis* in much the same way as it does in *E. coli*, there are important differences in (i) the proteins involved and (ii) their localisation within the cell. DivIVA protein is a key component of division site selection in *B. subtilis*. Our gel filtration analysis revealed that DivIVA forms oligomers. To examine the oligomerization state more carefully, we performed analytical ultracentrifugation and blue native polyacrylamide gel electrophoresis experiments. These showed that DivIVA exists predominantly as a 10-12 mer (7). The role of DivIVA and the min system in sporulation is not known. In other work, we have shown that the absence of active MinCD during sporulation causes the formation of thin sporulation-like septa at the mid-cell position (1).

The process of mid-cell division just described, is characteristic of vegetative growth and gives rise to two progeny cells of equal size. In contrast, during sporulation, the developing *B. subtilis* cell undergoes a process of asymmetric division in which the septum is formed near one pole of the cell. The switch in the localisation of FtsZ rings from medial to bipolar sites requires Spo0A, the master transcriptional regulator in sporulation. Our work led to the crystallisation of the two functional domains of Spo0A from *B. stearothermophilus* (6) and the solution of their structures by MAD phasing techniques (2-4). The N-terminal, receiver/phosphoacceptor domain (N-Spo0A) was crystallised in two different forms, in one of which the protein is phosphorylated. The crystal structures described above, together with results from biochemical, biophysical and genetic analyses of Spo0A and its domains suggest a model for the action of this important response regulator (5).

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## COMPUTER SIMULATIONS OF THE STRUCTURE OF POTENTIAL CHEMOTERAPEUTICS AND THEIR CONJUGATES WITH NUCLEIC ACIDS

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Antisense technology is a novel drug discovery method [1-2]. Each antisense drug is designed to bind to a specific sequence of nucleotides in its mRNA target to inhibit production of the protein encoded by the target mRNA.

Antisense drugs can be designed to treat a wide range of diseases including cancer. This method is expected to become more efficient than inhibition of resulting protein products by classical drugs. The oligonucleotides with natural chemical composition, the first candidates for the „antisense" drugs, have been, however, found as unsuitable for in vivo applications because of their insufficient resistance against nucleases.

I. Modified oligonucleotides [4]. Three undecamer complexes were analyzed: natural $dT_{11}.dA_{11}$ duplex as a reference and two its analogs with alternating modified and natural linkages in the deoxyadenosine chain. The isopolar, non-isosteric modified linkages were of 5'-O-$PO_2$-$CH_2$-O-3' (5'PC3') or 5'-O-$CH_2$-$PO_2$-O-3' (5'CP3') type. Explicit net-neutralizing $Na^+$ counterions were placed at the phosphates of the oligonucleotide by the EDIT module of AMBER 5.0 software package. The nucleic acid with the $Na^+$ counterions were surrounded by a periodic box of ~10000 TIP3P water atoms which extended approximately 10 (in each direction) from the nucleic acid atoms. The parameters described by Cornell et al [3] do not contain explicitly force constants needed to describe the modified parts of phosphonate analogs. Missing torsion force constants were gained by the same way as for the phosphodiester linkage in the original AMBER forcefield [3]. Fully solvated trajectories were computed by using the SANDER module of the AMBER 5.0 software package. The 2.5 ns MD runs have fully confirmed the stability of the phosphonate structures.

II. Modified dinucleotides [5]. It seemed advantageous to test the properties of synthetic compounds at the level of dinucleotides, which are readily obtainable with a wide variety of linkage modifications. Four model structures representing dodecamer triplex chains have been studied. Each model system consisted of two mutually antiparallel uridine dodecamers and a pseudostrand consisting of six A-dimers bound by Watson-Crick and Hoogsteen hydrogen bridges to the first and second $rU_{12}$ strands ($rU_{12}(ApA)_6rU_{12}$, $rU_{12}$(A5'PC3'A)$_6rU_{12}$, and $rU_{12}$(A5'CP 3'A)$_6rU_{12}$). The main significant differences between the investigated complexes were found in the stability of the Hoogsteen hydrogen bridges. Except for the $rU_{12}$(A5'CP3'A)$_6rU_{12}$ complex, several Hoogsteen hydrogen bonds for the inner adenosines in average MD structures exceeded limit of 2.1 Å.

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## USE OF VIBRATIONAL SPECTROSCOPY FOR STUDY OF BIOMOLECULES

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In our contribution we focus on applications of the methods of infrared (IR) and Raman spectroscopy in structural analyses of proteins and nucleic acids. The vibrational spectroscopic methods do not yield directly the three-dimensional structures of proteins and nucleic acids. However, the local structures of functionally important groups within a macromolecule, and changes in these local structures that may relate to biological functions, are conveniently probed by vibrational spectroscopy. Experimentally, optical spectroscopic methods are highly versatile, being applicable to a number of different sample morphologies and over wide ranges of sample temperature, concentration, and solvent environment. This approach is also well suited to probing an extensive range of biodynamical processes with time scales ranging from $10^{-13}$ s up to days.

Raman and IR spectroscopy for protein and nucleic acid structure analysis have the following advantages:

Both techniques are nondestructive and samples may be recovered and assayed for biological activity after spectroscopic examination.

Raman and IR methods are applicable to samples of virtually any morphological form including aqueous and non-aqueous solutions, precipitates, gels, films, fibers, single crystals, powders and amorphous solids. Data are generally transferable from one morphological state to another of the same sample. This has important practical benefits e.g. in comparing the molecular structure of a protein in the crystal with that prevailing in solution.

A small sample volume is required (typically 10 μl is sufficient for Raman spectroscopy and 20 μl for IR spectroscopy).

Both Raman scattering and infrared absorption processes occur on a time scale that is very short (≈ $10^{-15}$ s) in comparison to time scales of fluorescence (≈ $10^{-9}$ s) and magnetic resonance (≈ $10^{-6}$ s). Thus, in time-resolved studies vibrational spectroscopy can sample fast biological processes inaccessible by other techniques.

There exists a large database of IR and Raman data of proteins, nucleic acids and their constituents for which reliable spectra-structure correlations have been established.

Relatively high Raman intensity comes from molecular vibrations inducing a large change in molecular polarizability. For proteins, Raman spectra are dominated

by bands assignable to main chain peptide groups, aromatic side chains (Trp, Phe, Tyr), sulfur containing side chains (Met, Cys) and side-chain carboxyls. These bands are often sensitive to local environment and can serve as local probes of side-chain environment.

Structural interpretation of vibrational spectra is based on conformation markers. Vibrational spectral bands constitute a detailed and unique fingerprint of the molecule. For complex molecules, the vibrational modes and their corresponding spectral bands can neither be assigned directly to the atomic displacement coordinates, nor readily calculated from them. Thus the vibrational spectrum associated with a particular molecular structure cannot be exploited as a fingerprint of that structure until it is first correlated with it by an independent method (structures determined by X-ray crystallography or NMR techniques can serve as a basis for such correlations). Since each band in the spectrum originates from the vibration of a specific group of atoms (normal mode of vibration) with well-defined geometric characteristics (known bond lengths, bond angles, …), the correctly assigned band will serve as an unequivocal indicator of the corresponding conformational feature.

Several examples illustrating usefulness of vibrational spectroscopy for structural biology can be found in other contributions from our group - R. Ettrich et al. (p. 35, 36), J. Hanuš et al. (p. 37), K. Hofbauerová et al. (p. 38), J. Kapitán et al. (p. 40) and K. Ruszová-Chmelová et al. (p. 47).

with encouraging results. Recently, large protein complexes such as 40S ribosome subunit, 26S proteasome, clathrin cage and ATP synthetase complex have been separated by blue native electrophoresis and analyzed in the ion trap. In studies of complete covalent structures of small proteins, we have established the primary structure of DQH sperm surface protein including the disulfide bonding and O-glycosylation at threonine 10 [3]. Finally, our long-term efforst to produce ligand-binding domains of natural killer cell activation receptors as recombinant proteins suitable for structural studies will be shortly summarized.

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## CHEMISTRY AND MASS SPECTROMETRY APPLIED TO PROTEIN MICROIDENTIFICATION AND STUDIES OF THE COMPLETE COVALENT STRUCTURE OF SMALL PROTEINS

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The investigations of our research group is based on protein chemistry and biochemistry, the use of chromatography and electrophoresis, protein sequencer with Edman chemistry, MALDI mass spectrometry and nanoLC-MS/MS on ion trap. These approaches have been applied to a large number of biomedical problems in three major research areas. In identification of unknown peptide and proteins, we participated in the studies of peptides active in celiac disease [1] and in microidentification of small amounts of proteins directly from gel spots. Moreover, both MALDI mass spectrometry and ion trap mass spectrometry have been used for mapping proteins within the activation and inhibitory membrane microdomains of natural killer cells [2]. Here, proteomics without gel has been applied extensively

## LIVE CELL AS A GEL-LIKE COLLOIDAL SYSTEM. MUSCLE CONTRACTION

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The growth of the self-assembled structure of carbon colloidal particles has recently been studied by experimental methods at Faculty of Electrical Engineering of Czech Technical University in Prague [1]. To induce different states of carbon self-assembly in the polymer melt, the samples were annealed at 190-200C for time periods up to several days. Impedance analyses *in situ* and *in real time* were measured during the process. Ionic concentration in the samples was checked by the Rutherford Back Scattering method (RBS). Optical microscopy, Atomic Force Microscopy (AFM), and Transient Electron Microscopy (TEM) were used to investigate the state of the clusters of carbon colloids.

Colloid theory only has provided an effective interpretation of the complex behavior of the self-assembled structure of carbon. Since 1998, we try to interpret the

metamorphoses of more general structures of *carbonaceous colloidal particles.* A more intricate hierarchy of construction can be invoked in the case of organic systems based on carbonaceous colloidal particles. Biological structures formed by self-assembly may include wide variety of structures in a cell. Their dependence on a dynamic equilibrium makes these architectures reversible and reorganizable under mild conditions.

The gel-like nature of the live cell forms the foundation of our study. We explore the possibility that the phase-transition of the gel could be a common denominator of cell function. Muscle cell function is chosen as an example of this behavior.

Self-assembly of the gel involves equilibrium of noncovalent interactions (mainly van der Waals forces and electrostatic forces) between the surfaces of particles. The first part of the contribution is a short introductory to this problem of the colloid chemistry [2].

The second part describes shortly classical contractile mechanism of muscle cells. In this mechanism, the actin-binding protein myosin is the molecular motor that converts the energy of ATP hydrolysis into movement of one cellular component along another [3].

The third part of the contribution explains the effect of van der Waals forces and electrostatic forces on the function of sarcomere in a muscle cell. The muscle twitch is interpreted as a phase transition of the lattice of myosin heads.

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## PROTEIN ENGINEERING OF DEHALOGENATING ENZYMES

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Haloalkane dehalogenases are bacterial enzymes cleaving the carbon-halogen bond of the halogenated aliphatic compounds by a hydrolytic mechanism. These enzymes have a potential application in detoxification of subsurface pollutants and recovery of industrial side products . Modification of the substrate specificity and activity of these enzymes is required for optimisation of their catalytic properties.

This presentation will introduce an on-going research project focused on structure-function relationships and engineering of haloalkane dehalogenases and gamma-hexachlorocyclohexane dehydrochlorinase. The major objectives are: (i) to understand the structural determinants of catalytic activity and substrate specificity of these enzymes, (ii) to design mutant proteins with modifies catalytic properties, (iii) to construct these mutants using DNA-recombinant technologies and (iv) to characterize them structurally and functionally.

(i) Comparative studies of primary sequences and tertiary structures are being performed for identification of key amino acid residues for mutagenesis studies [1, 2] . The primary sequences are known for twenty six different haloalkane dehalogenases, while the three-dimensional structures are know for three family members. Novel genes of haloalkane dehalogenases were biochemically identified in bacteria [3] and newly employed molecular biology techniques should make the gene hunting even more efficient. Eight new structures of enzyme-substrate and enzyme-inhibitor complexes were determined by the protein crystallography [4].

(ii) Molecular docking followed by energy minimization, energy partitioning and multivariate statistical analysis is being used to study the binding of different substrates into the enzyme active site [5]. Quantum-mechanic calculations are being applied for the modelling of the biochemical reactions in the active site [6-8] and for study of the effect of substitutions on transition state and product stabilization [9]. Molecular dynamic simulations are being used provide information about the functionally relevant motions [10].

(iii) Mutant proteins are being constructed by site-directed mutagenesis and DNA-shuffling techniques. The later methods are being applied on a set of genes of haloalkane dehalogenases with the attempt to obtain hybrid enzymes with entirely novel catalytic properties. Until today, more then forty four point mutants and twelve hybrids of the haloalkane dehalogenase LinB [11] and thirty two point mutants of gamma-hexachlorocyclohexane dehydrochlorinase have been constructed [12, 13].

(iv) Protein variants are functionally compared with the wild type enzymes using steady-state and pre-steady state kinetic analysis and substrate specificity analysis; and structurally characterized using CD-spectroscopy and X-ray crystallography.

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# INFLUENCE OF HEMOGLOBIN - HAPTOGLOBIN INTERACTION ON THE CHAPERONE-LIKE ACTIVITY OF HUMAN HAPTOGLOBIN

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Human haptoglobin (Hp) belonging to the group of serum alpha - 2 - glycoproteins is known to have chaperone-like activity in preventing thermally induced aggregation of gamma-crystallin and other proteins [1]. It was shown that Hp prevents aggregation by providing appropriately placed hydrophobic surfaces. Structural perturbation of Hp is enhancing its protection ability. Human hemoglobin (Hb) was chosen as substrate because it can form a very tight complex with Hp. Thus the Hb-Hp complex formation together with its chaperone-like activity would probably give additional information to elucidate the molecular mechanism of the chaperone action.

The influence of Hb-Hp complex formation on the chaperone-like activity of Hp was investigated in the process of thermal aggregation of catalase and hemoglobin by spectrophotometric and spectrofluorimetric methods. Additional conformational changes, observed after Hb-Hp complex formation, led to variations of chaperone activity. After sufficiently long time the protection ability of Hb-Hp complex disappeared and even a kind of anti-chaperone activity was arising. On the basis of experimental data, sequence comparison and knowledge-based molecular modelling [2] two chaperone binding sites on each Hp heavy chain can be specified. The functional non-identity of these sites can explain the observed chaperone and anti-chaperone activity of Hb-Hp complex.

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## BEHAVIOUR OF THE CHAPERONE-LIKE PROTEIN HAPTOGLOBIN UNDER HEAT SHOCK CONDITIONS

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Human haptoglobin (Hp), belonging to the group of serum α-2-glycoproteins, is known to have chaperone-like activity in preventing thermally induced aggregation of enzymes and lens proteins [1]. Haptoglobin of the genetic type 1-1 is composed of two heavy chains (H chains), which are connected with two smaller light chains (L chains) to give a tetrameric protein of 85 kDa. A model of the heavy chain of haptoglobin was proposed, based on its sequence homology with serine proteases [2]. Experimentally and by means of molecular modeling the existence of two chaperone-binding sites on each heavy chain was shown [3].

In this study we examined the behaviour of haptoglobin itself under heat shock conditions. Optical density at 360 nm was used to measure the influence of heat shock on the protection ability. Fluorescence of ANS was measured for examining hydrophobic properties of Hp. Disulfide-bridges and behaviour of Trp and Tyr were investigated by Raman spectroscopy. Changes in secondary structure during heat shock were followed by circular dichroism, Raman and FTIR spectroscopy.

The protein, once exposed to elevated temperature, did not completely return to its original conformational state but adopted a conformation which was characterized by markedly enhanced surface exposure of hydrophobic sites. We have found that the conformational transition upon heating, which lacked reversibility and was associated with the increase in surface hydrophobicity, was accompanied by an increase in ordered secondary structure, especially α-helical structure. Disulfide bonds were not influenced by heating and thus the tetrameric structure of the molecule seemed to be preserved.

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## COMPUTER MODELING AND SIMULATIONS OF NUCLEIC ACIDS AT NCBR

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A brief overview of the methods utilized for the research on structure and dynamics of nucleic acids will be given. The main aim of the projects carried out in the Center is a study of conformational behavior and its relationship to the function of nucleic acids.

Methods of conformational search and molecular dynamics (MD) have been applied on the range of DNA and RNA structures. We have studied fragments of DNA (heptamer, octamer), nucleotide sugars, noncanonical structures as DNA quadruplexes, complexes DNA with drugs or with proteins. A long scale molecular dynamics has been applied together with a post processing analysis including the study of solvation, interactions with ions, the free energy contribution and the entropy analysis. MD has also been used to refine structures proposed by NMR spectroscopy - together with restrains obtained by NMR it provides an information about the 3D geometry of the molecules.

Biologically interesting molecules including the complexes have been studied by MD which started from structures experimentally observed by X-ray crystallography. Additional simulations have been carried out on mutated structures, whose 3D geometry has not been known, in order to examine the influence of the mutation on the conformational behavior. Advanced simulation methods, such as Locally Enhanced Sampling or Thermodynamic Integration, have also been applied.

The results of the simulations have been carefully analyzed with the help of wide range of methods. Novel approaches to calculate the solvation energy are tested and they provide more detailed view into the structure and dynamics of nucleic acids.

## MOLECULAR RECOGNITION AND MISRECOGNITION IN COMPLEX MOLECULAR SYSTEMS

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Molecular Specificity is a central issue in biology. The mechanism by which nucleic acid sequence information (the gene level) is translated into amino acid sequence information (the protein level) is based on specific base pairing and is well known as the genetic code. It is generally accepted, that the three dimensional conformation of a protein as a major determinant of its function is determined by its amino-acid sequence. However, it is not known whether a general protein folding code, relating protein sequence and conformation exists and not fully understood, how a nascent polypeptide chain can find the native conformation

in the course of protein folding on a biologically meaningful time scale. Our knowledge about this ´second half of the genetic code´ must, therefore, be regarded as being rather descriptive rather than as an understanding based on mechanism and theory. It is generally believed, that function arises from structure. Structural complementarity between reaction partners is crucial for the capability of a protein or enzyme to recognise its substrates, natural inhibitors or modulators of biological activity. An understanding of molecular specificity is important also from the applied point of view, especially with regard to a rational design of agents for therapeutic intervention. The most broadly characterised example for molecular recognition are the protein inhibitors of serine proteases, occurring in the plant and the animal kingdom. The function of these inhibitors is to prevent an overshoot of endogenous or exogenous proteolytic activities resulting in fatal disorders.

We have combined protein engineering with structural biology for studying the mode of action of these inhibitors and the specificity of their interactions. Our main concern were perturbations of functionality and specificity in the mutants. Having this problem in mind, we have selected the double-headed Bowman-Birk-inhibitor (sBBI) of trypsin and chymotrypsin from soybean as a model protein. Mutations have been introduced into the first sub-domain (directed against trypsin because there is an active-site lysine in position 16) while using the second sub-domain (directed against chymotrypsin via an active-site leucine in position 43) as an internal standard for a fast and sensitive detection of irregularities in the molecule induced by mutations. A synthetic gene coding for a recombinant Lys16Arg and Met27Ile variant of the parent protein (termed as rBBI) was synthesised, expressed as a fusion protein with a truncated beta-galactosidase, reduced under denaturing conditions after cyanogen bromide cleavage and refolded into the fully active form. The three dimensional structure of sBBI in the free form and in the complex with bovine trypsin were determined by molecular replacement. The structure of the free inhibitor reveals structural peculiarities such as polar residues and embedded water molecules in the interior and exposed hydrophobic patches on the molecular surface. This ´inside-out´ situation is reminiscent of the situation thought to be present in partially folded proteins. The binding of sBBI and rBBI to trypsin and chymotrypsin was monitored by means of titrations and the catalytic turnover was studied by means of high performance capillary electrophoresis in conjunction with protein sequencing and mass spectrometry. These studies reveal a narrow specificity for rBBI reacting with trypsin via the first and with chymotrypsin via the second subdomain, resulting in a stoichiometric 1:1-ratio with the two enzymes as expected. In contrast, the second sub-domain of the natural inhibitor displays a broadened specificity, which can react with trypsin in contrast and with chymotrypsin in accord with primary specificity. This unexpected behaviour of sBBI is also underscored by an unexpected proteolysis of the soybean inhibitor at Leu43 in addition to Lys16. The broadened specificity of the second subdomain was also proven by crystallographic analysis showing that the inhibitor binds to trypsin not only via Lys16 as expected but also via Leu43 in contrast to primary specificity. This broad specificity of the second binding site appears as a result of close hydrophobic contacts with the surface loops surrounding the active site of the enzyme formed by the critical Met27 residue and other hydrophobic residues outside the principal binding area of the second subdomain of the inhibitor. Obviously, the regular preference of trypsin for basic amino acid residues dominated by salt bridges is energetically unfavourable when the primary specificity area is surrounded by hydrophobic residues occluding the active site from water.

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## THE CONFORMATIONAL COMPATIBILITY OF THE ApA DIMER WITH POLY(rU) IMPROVED BY THE –$CH_2$– LENGTHENING OF THE DIMER INTERNUCLEOTIDE LINKAGE – A 2D RAMAN ISOTHERMAL STUDY

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The new procedure of processing the 2D Raman data was used to investigate the hybridisation and structural properties of the complete family of ApA phosphonate analogues lengthened by insertion of the –$CH_2$– group. It provided direct information about the formation, stoichiometry and concentration of the complexes formed in mixed samples of modified A-dimers and poly(rU). Raman spectra of the complexes were isolated, providing information about their structure.

Except for the conformationally restricted ACHpA(2'3'*endo* > 5), all the analogues form triplex-like complexes with poly(rU) at room temperature, where two polymer strands are bound by Watson-Crick and Hoogsteen bonds to a central pseudostrand consisting of A-dimers. For all these modifications, the overall conformation of the triplexes was found to be similar, and the first poly(rU) strand and the pseudostrand of dimers joint by Watson-Crick bonds adopt a conformation typical for the A-form of RNA duplex, according to their extracted Raman spectra.

A simple semi-empirical model was introduced to describe the observed decrease of the A-dimer binding efficiency when adenosine concentration decreases.

Apparently, for most of the analogues studied (including the natural 3'-5' phosphate linkage), the enthalpy decrease of the initiation step (i.e. when two polymer strands are joint together by one dimer) is not sufficient to balance the entropy change of the system. Therefore the creation of a stable complex at room temperature requires the formation of a central pseudostrand chain consisting of several A-dimers.

For $Ap^{CH2}A$-dimers (the methylene group is inserted closer to the 5' position), the initial binding conditions are much more favorable, in terms of enthalpy, as compared to other modified or natural linkages. MDS indicate that these differences are consistent with a good compatibility of the three-dimensional conformation of this phosphonate linkage toward the second (Hoogsteen) poly(rU) strand. There is no additional hydrogen bond between the A-dimer linkage and the poly(rU) ribose (as for ApA 3'-5') and no sterical conflict between the methylene group and uracil (as for $A^{CH2}pA$ 3'-5'). Although these properties do not directly advantage $Ap^{CH2}A$ linkages for the duplex formation with the natural nucleic acids, they can serve as an indication of the suitable structures enabling good interactions with other molecules over the major groove.

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## SECONDARY AND TERTIARY STRUCTURE OF THE NUCLEOTIDE-BINDING DOMAIN OF THE α-SUBUNIT OF $NA^+/K^+$-ATPASE

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$Na^+/K^+$-ATPase (EC 3.6.1.37), member of the P-type ATPase family, is an integral membrane protein, which transports sodium and potassium ions against an electrochemical gradient. This enzyme consists of two subunits, the catalytic a-subunit, which carries out all transport and catalytic functions, and the associated β-subunit. Obsil et al. [1] have expressed the large cytoplasmic loop and showed that the construct is able to bind nucleotide triphosphates.

We have prepared the nucleotide-binding domain of this large cytoplasmic loop as a recombinant protein containing the ATP-binding site. The expression vector pGEX-H4-H5 was constructed by inserting the cDNA fragment of mouse brain $Na^+/K^+$-ATPase into pGEX-2T multiple cloning site at the BamHI and EcoRI site location downstream from GST coding sequence. The ligated DNA was transformed into competent *Escherichia coli* DH5a cells. Fusion protein was cleaved using thrombin and purified by HPLC. The deduced amino acid sequence shows high homology to the $Ca^{2+}$-ATPase, which allowed the construction of a model structure using the MODELLER program. Refinement was achieved through algorithmic analysis and minimization with SYBYL. We were able to specify the ATP-binding site by docking of ATP into the active site with the AUTODOCK program. The model structure has been confronted with the estimation of secondary structure from the experimental far-UV CD spectra (182–260 nm) – by deconvolution using a combination of singular value decomposition and variable selection, additionally a backpropagation network model was used; and Raman spectra (the amide I band) – by reference intensity profiles method. The influence of nucleotide binding on the secondary structure of the N-domain was also observed by far-UV CD and Raman Spectroscopy. The secondary structure determined by far-UV CD and Raman spectra correspond to our model structure and thus support on the one hand the quality of the model and on the other hand the correct folding of the recombinant protein.

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## NMR STRUCTURAL STUDIES OF PROTEINS FROM MASON-PFIZER MONKEY VIRUS

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Mason-Pfizer monkey virus (M-PMV) was isolated for the first time from a mammary tumor of a female rhesus monkey (Chopra et al., 1970). The virus belongs to a family of RNA viruses, which is characterized by the transcription of its genome from the diploid RNA to the corresponding DNA through the activity of the reverse transcriptase. Although it is known that M-PMV is not directly responsible

for the Simian Acquired Immuno-Deficiency Syndrome (SAIDS, the simian equivalent of AIDS), it exerts T-cell immunosuppresive effect (Fine et al., 1975). We have focused our attention to two proteins encoded by this virus. The first one is protease, a key enzyme for the maturation of viral particles. It has been discovered that M-PMV PR occurs in three active forms with the molecular weight of 17 kDa, 13 kDa and 12 kDa per monomer, respectively (Zábranský et. al. 1998). While the specificity of all forms is identical, the activity drops with the shortening of the amino acid sequence. However, it turns out that the 12 kDa form is similar to the other, fully active members of this family (HIV1, RSV...) in terms of the length of the amino acid sequence and the global fold.

Our goal is to elucidate the influence of the C-terminal extensions of both longer forms on the increased activity of this protease. Therefore, we have started the process of determination of three-dimensional structure of the 12 kDa form of M-PMV protease by a combination of isotopically-aided NMR spectroscopy and computational methods. In the next step we will extend this study to the 13 kDa form to find out the definition of the extended C-terminus.

M-PMV is a prototype of a so-called D-type subfamily of retroviruses conferring a highly specific morphogenesis. While the C-type retroviruses (HIV-1, SIV...) assemble immature capsids at the inner side of the plasma membrane of a host cell, the D-type viruses preassemble them within the cytoplasm and then they are transported to the cell membrane for budding (Sommerfelt et al., 1992). However, it was discovered that a tiny modification of the sequence of the matrix protein of M-PMV causes large changes in the lifecycle of the virus (Rhee et al. 1990). For example, point mutation of Arg 55 for Trp or Phe results in reversion of the type of assembly from D-type to a C-type, i.e. capsids are assembled at the plasma membrane instead of the intracytoplasmic assembly typical for M-PMV. The project that we have recently launched should provide us with an answer to the following question: Are the changes of morphogenesis of Mason-Pfizer monkey virus caused only by local structural changes of the matrix protein or by larger changes of the global fold of this protein? Within the frame of the project various mutants of the matrix protein will be subjected to comparative structural studies.


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# COMPUTER MODELLING OF MOLECULAR INTERACTIONS


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Molecular modelling techniques are widely used in fundamental research as well as in the chemical, pharmaceutical and agrochemical industries. Our work is concentrated on the computer drug design with utilizing additional knowledge of chemical and biochemical properties of the compounds under study.

Despite the fact that computer modelling usually does not reach a proper lead directly, many unrealistic models can be eliminated in this way before expensive and time consuming chemical synthesis is started. The docking problem and Gibbs energy calculation are the most demanding steps in molecular modelling and molecular simulation. Thermodynamic integration and perturbation methods in connection with the thermodynamic cycle are promising approaches; however, simple estimates are sometimes of the same power and speed up the calculations significantly. Molecular dynamics combined with additional restraints and constraints can be used to predetermine expected mutual orientation of molecular fragments during the docking manoeuvre. Special potential functions applied in the calculation are based on chemical properties and molecular structure of close analogs of the modelled compounds. Slow heating followed by annealing are an important part of the procedure.

Two areas of application of these procedures are demonstrated: interaction of enzymes with substrates (cathepsin B - polymeric anticancer drugs) and receptors with hormones (insulin receptor – insulin analogs).

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## A STUDY OF MAJOR URINARY PROTEIN I (MUP-I) USING NMR SPECTROSCOPY

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Investigation of the protein content of mouse urine has shouwn that it consists protein termed the Major Urinaty Proteins (MUPs). These are acidic proteins with molecular masses of approximately 19 kDa. The MUPs are associated with pheromonally active ligands including relatively tightly bound 2-sec.-butyl-4,5-dihydrothiazole. MUPs are proteins binding small, hydrophobic ligands that are known to posses the capability of chemical signaling and they have been assigned to the lipocalin superfamily of proteins. The lipocalins have a distinct tertiary structure consisting of eight, nine or ten beta-sheeds arranged in beta-barel to form a central hydrophobic pocket or calyx into which the ligand is inserted. Current opinion looks for a role for the MUPs in either the timed release of odorants or in their protection from oxidation.

In the initial stage of any investigation by NMR spectroscopy, each resonance frequency must be associated with specific nucleus. 2D HSQC experiment is measured at first. Number of the peaks responds to number of the amino acids in this spectrum. Acquirement of 3D HNCACB and CACB(CO)NH spectra is necessary for backbone assignment, which is sequence-specific. TOCSY-HSQC, HCCH-TOCSY, C(CO)NH and H(COO)NH experiments are using for the side chain assignment. Assignment of backbone has been completed and actually, we are finishing side chain assignment.

In the second stage of investigation by NMR spectroscopy is necessary to determine interatomic distances and dihedral angles. These parametres are obtained from volumes of crosspeaks from the NOE experiments, and 3J coupling constants, respectively. A set of spectra providing structure information has been recorded. This set includes 13C/15N edited-NOESY, HNCA[CB]-E.COSY, HNCA[HA]-E.COSY, HNCA[CO]-E.COSY, HNCACB-[CO]-E.COSY. Currently, structural parameters including NOEs, 3J(HNCbeta), 3J(HNHalpha), 3J(HNC'), 3J(CbetaC'i-1)are being extracted from the spectra.

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## CONFORMATIONAL STUDIES OF ZWITTERIONIC AMINO ACIDS BASED ON THE RAMAN OPTICAL ACTIVITY

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Raman optical activity (ROA) provides spectra significantly more sensitive to molecular conformation than non-polarized scattering or absorption. The interpretation of experimental data is dependent on ab initio modelling of vibrational frequencies and spectral intensities. For larger molecules the density functional theory can be conveniently used for precise computations of second energy derivatives as well as for electromagnetic tensors needed for simulations of spectral intensities. The *ab initio* interpretation, however, is difficult because of the conformational flexibility, strong interaction of zwitterionic molecules with solvent and inadequacy of usual vacuum models.

Our goal was to find models suitable for simulation of spectra of simple amino acids L-Ala, L-Pro, L-Ser and L-Cys. In an attempt to improve harmonic vibrational frequencies, we used a combination of the modern B3LYP and BPW91 functionals with the COSMO model of solvent. We found that the ROA intensities are very sensitive to conformational changes as well as that some spectral features can be explained only by presence of several conformers.

We have also investigated conformational space of L-alanine in detail rotating the $CH_3$, $NH_3^+$ and COO groups. Dependence of the ROA spectra of zwitterionic L-alanine simulated with a Becke3LYP/6-31G* calculation and the COSMO solvent model on the rotation of the $NH_3^+$ group ($\angle C-{}^{\alpha}C-N-H$) is depicted in Fig 1. The spectra suggest that the $NH^{3+}$ group is, unlike the $CH_3$ and COO groups, freely rotating.

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## COMPARATIVE BINDING ENERGY ANALYSIS OF THE SUBSTRATE SPECIFICITY OF HALOALKANEDEHALOGENASE FROM *Xanthobacter autotrophicus* GJ10

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Comparative binding energy (COMBINE) analysis is the computational technique for deriving quantitative structure-activity relationships from the set of 3D structures of enzyme-substrate complexes.

COMBINE analysis was conducted for 18 substrates of the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 (DhlA): 1-chlorobutane, 1-chlorohexane,dichloromethane, 1,2 - dichloroethane, 1,2-dichloropropane, 2-chloroethanol, epichlorohydrine, 2-chloroacetonitrile, 2-chloroacetamide, and their brominated analogues. The purpose of the COMBINE analysis was to identify the amino acid residues determining the substrate specificity of the haloalkane dehalogenase.

This knowledge is essential for the tailoring of this enzyme for biotechnological applications. Complexes of the enzyme with these substrates were modeled and then refined by molecular mechanics energyminimization. The intermolecular enzyme-substrate energy was decomposed into residue-wise van der Waals and electrostatic contributions and complemented by surface area dependent and electrostatic desolvation terms. Partial least-squares projection to latent structures analysis was then used to establish relationships between the energy contributions and the experimental apparent dissociation constants. A model containing van der Waals and electrostatic intermolecular interaction energy contributions calculated using the AMBER force field explained 91% (73% cross-validated) of the quantitative variance in the apparent dissociation constants. A model based on van der Waals intermolecular contributions from AMBER and electrostatic interactions derived from the Poisson-Boltzmann equation explained 93% (74% cross-validated) of the quantitative variance. COMBINE models predicted correctly the change in apparent dissociation constants upon single-point mutation of DhlA for six enzyme-substrate complexes. The amino acid residues contributing most significantly to the substrate specificity of DhlA were identified; they include Asp124, Trp125, Phe164, Phe172, Trp175, Phe222, Pro223, and Leu263. These residues are suitable targets for modification by site-directed mutagenesis [1].

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## MOLECULAR MODELING OF PEPTIDES AND PROTEINS AT NCBR

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X-ray crystallography, NMR spectroscopy, and neutron diffraction are typical experimental methods to analyze biomolecules at the atomic level. High-resolution structures are usually preferred for a reliable analysis of structural aspects of small molecules associated with a host protein [1]. In the absence of high-resolution experimental data, theoretical studies, such a molecular dynamics simulations, represent complementary methods to understand the dynamics and energetics of interactions of small molecules associated with proteins.

It will be discussed the projects solved at NCBR using molecular dynamics methods applied to peptides and proteins.

The first project will be molecular dynamics study of phosphotriesterase, the protein that catalyzes the hydrolysis of wide range organophosphate insecticides and chemical warfare agents. The X-ray structure of phospho triesterase [2] has been solved, but the chemical mechanism of phosphate hydrolysis and the role of amino acids in the active site has not been explained yet. The molecular dynamics could help explain the reaction mechanism of hydrolysis.

The second project will be MD study of alpha-1,4-galactosyltransferase, enzyme that catalyses the synthesis of lipopolysaccharides in bacteria Neisserii meningitis [3]. These lipopolysaccharides ensure protection of bacteria against host immunity system. The mechanism of synthesis is not known yet.

The third discussed project will be the study of cyclin dependent kinase cdk2 using molecular dynamics methods. The enzyme cdk2 plays a key role in cell cycle [4]. The inhibitors of the enzyme are potential cancer therapeutics[5]. The special attention will be devote to study of the tightly bound water molecules [6-8]

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## STRUCTURAL BIOLOGY OF TELOMERES

### Tatiana Kubičárová and Jiří Fajkus

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Telomeres are nucleoprotein complexes, which are involved in important biological processes as replication, transcription and recombination. Their presence is critical for stability of chromosomes. Telomeres in most eukaryotic organisms lengthen by the action of telomerase, a ribonucleoprotein complex with reverse transcriptase activity. Telomerase is strongly processive enzyme and, therefore, its activity must be controlled. The regulatory mechanisms of telomere lengthening are mediated by the expression and interaction of telomerase subunits and by telomere-binding proteins.

Our laboratory is focused on nucleoprotein complexes of chromosome ends. We have observed developmental and tissue specific regulation of telomerase activity and telomere lengths in two model plants (Říha et al., 1998, Fajkus et al., 1998). More detailed studies showed the inhibition of telomerase due to telomere-binding proteins from nuclei of telomerase-negative tissues (Fulnečková and Fajkus, 2000). Currently, we continue characterisation of selected telomere-binding proteins which includes their chromatographic purification, and MS analysis. The next step will be a structural analysis of telomere-binding proteins and their binding to DNA.

Another important aim of our studies is characterisation of terminal and subterminal DNA and chromatin structure (Horáková and Fajkus, 2000, Sýkorová and Fajkus, 2001, Fajkus and Trifonov, 2001).

Besides the above "fundamental research", we are involved in research of telomere dynamics in human malignant cells (Krejčí and Koch, 1999, Maláska et al., 2000) as a useful diagnostic marker.

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## CRYSTALLIZATION OF MEMBRANE AND NON-MEMBRANE PROTEINS AT UNIVERSITY OF SOUTH BOHEMIA AT CESKE BUDEJOVICE

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The Laboratory of Crystallogenesis has been built at the Institute of Physical biology. Nowadays, three different proteins are available for crystallization experiments. The pokeweed antiviral protein (PAP) from *Phytolacca acinosa* and the tryptophan (W)-repressor binding protein *A* (WrbA) are non-membrane proteins and the five-chlorophyll reaction center of photosystem II from *Pisum sativum* belongs to the class of membrane proteins.

The antiviral protein, PAP*aci*, isolated from seeds of the Chinese pokeweed plant *Phytolacca acinosa*, is a single-polypeptide chain ribosome-inactivating protein (RIP). PAP acts as an N-glycosidase, which catalyzes the depurination of a single adenine base (A4324) in the sarcin/ricin domain of the large rRNA species in eukaryotic and prokaryotic ribosomes. This catalytic depurination of the α-sarcin loop impairs the interaction between ribosomes and elongation factor 2 (EF-2, EF-G in bacteria), resulting in the cessation of protein biosynthesis at the EF-2-mediated translocation step. PAPs effectively inhibit the replication of several plant and animal viruses such as potato and tobacco mosaic viruses, herpes simplex, influenza and HIV-1 viruses [1-2] and have been tested as a safe prophylactic agent [3].

The antiviral protein, PAP*aci* from *Phytolacca acinosa* is similar to the well-characterized antiviral protein from *Phytolacca americana* [4] but exhibits a significantly higher activity against plant virus infections. The isolated enzyme from seeds was purified and crystallized. SDS PAGE of pure material revealed two bands close to one another while material from re-dissolved PAP*aci* crystals consisted solely of the high MW band. Diffraction data were measured on beamline X13 (EMBL Hamburg) using a wavelength of $\lambda = 0.803$ Å. The crystals have space group I222 and cell parameters a = 78.63, b = 84.19, c = 90.88 Å. The asymmetric unit consists of one molecule. The crystal structure of PAP*aci* was determined at 1.7Å resolution by molecular replacement based on the structure of PAP from *Phytolacca americana* [4] (PDB ID: 1PAF).

The tryptophan (W)-repressor binding protein *A*, WrbA, is polypeptide that specifically binds a tryptophan

repressor protein (TrpR) which negatively regulates the transcription of genes of the tryptophan regulon in the excess of L-tryptophan [10]. Sequence analysis and homology modeling identified the WrbA as the member of new class of flavodoxin-like proteins with typical α/β twisted open-sheet fold but with a short well conserved insertion unique to this new family. The protein binds FMN specifically, weakly than many flavodoxins. The WrbA has no influence on the affinity or form of DNA binding by the TrpR, so its physiological role is still unclear [11].

The protein WrbA was expressed in *E. coli* and purified using the DEAE-cellulose and Affi-Gel Blue affinity columns equilibrated with 20 mM Tris and 20-mM sodium phosphate buffers. Columns were developed with a linear gradient of NaCl in mentioned buffers. Pure WrbA apoprotein was yielded after 50 % ammonium sulfate precipitation of Affi-Gel Blue column extract. 10-mg/ml WrbA protein has been used for crystallization experiments.

Photosystem II (PSII) is a large pigment-protein complex located in the photosynthetic membrane of green plants, algae and cyanobacteria. PSII is responsible for the photochemical splitting of water into protons, electrons and molecular oxygen by utilizing sunlight. For its central role in bio-energetic, PSII has been the subject of many crystallization attempts [5-7].

The complex of photosystem II of higher plants consists of the reaction center proteins D1 and D2, α- and β-subunits of cytochrome b-559, two chlorophyll-binding internal antenna proteins CP43 and CP47 and the complex of manganese-stabilizing proteins of 33, 23, 16 kDa. While the reaction centers and the internal antennae are formed by membrane proteins, the manganese-stabilizing complex consists of membrane extrinsic proteins.

This five-chlorophyll reaction center of photosystem II that have been used for the crystallization experiments was isolated from pea (*Pisum sativum*) and purified according to Vacha [8]. 15-mg/ml (1.3-mg/ml chlorophyll *a*) protein has been mixed with different type of detergents and additives. The commercial solutions for membrane protein screening and solutions exactly prepared by us as well were experimentally tested. Processes inside the drops have been observed during the period of 2-4 weeks at 289 K and 4-6 weeks at 277 K. The major part of drops contained precipitates, separated phases or segregated carotenoids in various forms. The acceptable pH rate ($7.00 \pm 0.50$) and the type of precipitant (PEG4K, PEG6K) have been already found. The crystallization experiments have been performed either in the absence or in the presence of additional amphiphiles (heptanetriol) that has been used to modify detergent characteristics and to facilitate membrane protein crystallization [9]. Nowadays crystallization experiments are still in the progress.


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## APPLICATION OF MM-PBSA FUNCTIONALITY IN AMBER TO THE BINDING OF INHIBITORS TO HIV PROTEASE

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Interactions of HIV-1 protease and its resistant mutants with its inhibitors have been studied. The inhibitor set comprised the clinically used ones as well as a subnanomolar inhibitor designated QF34 (Figure 1) (Konvalinka et al., 1997). The complex of QF34 with HIV protease is depicted at Figure 2.In the study, we aim at calculating binding free energies of protease-inhibitors complexes and correlate them with experimentally determined inhibition constants (Weber J et al., manuscript in preparation). We have been developing a protocol to model structures of resistant mutants of HIV protease to be able to predict the binding energy for the complexes structures of which have not yet been solved.

Molecular dynamics (MD) is a very powerful computational method which in conjunction with other techniques, may be exploited to calculate binding free energies between a protein molecule and its ligand (reviewed in Wang et al., 2001). One of such techniques is called MM-PBSA (molecular mechanics-Poisson Boltzmann surface area)

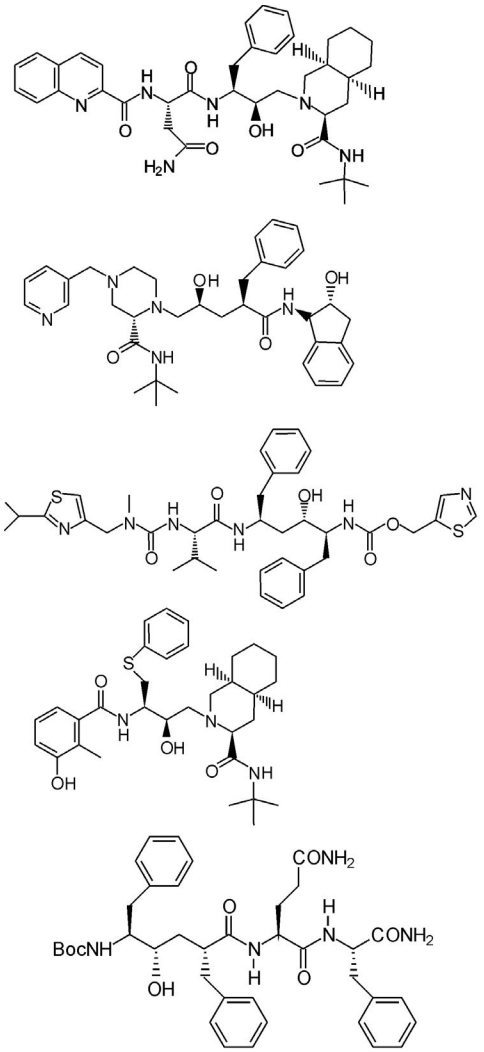

**Figure 1.** Inhibitors of HIV-1 protease: saquinavir, indinavir, ritonavir, nelfinavir, and QF34

and is implemented in the molecular simulation program package AMBER.

MM-PBSA is a computationally efficient and a less rigorous method for estimating binding free energies with reasonable accuracy. It consists of postprocessing of snapshots from molecular dynamics (MD) simulations. To calculate binding free energies ($\Delta G_{bind}$), it combines molecular mechanical interaction energies ($\Delta E_{MM}$) with solvation terms based on implicit solvation models ($\Delta G_{solv}$) and with entropy contributions($-T\Delta S$):

$$G_{bind} = E_{MM} + G_{solv} - TS$$

The MM-PBSA approach has been used to address a variety of tasks. These reached from the investigation of duplex and hairpin nucleic acid stability via protein folding problematic to studies on the strength of binding of various ligands and its analogues to their protein targets or their mutants. (reviewed in Kollman et al., 2000)

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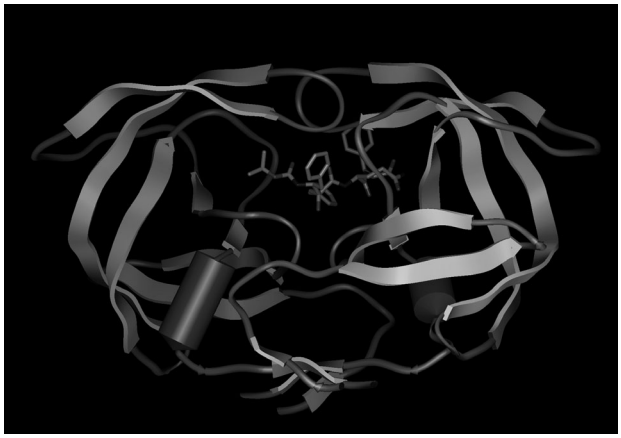

**Figure 2.** Complex of QF34 inhibitor bound to HIV protease (picture based on crystal structure determined at Rolf Hilgenfeld's laboratory at Institute of Molecular Biotechnology, Jena, Germany)

## DIMER-INDUCED SIGNAL PROPAGATION BY *BACILLUS SUBTILIS* SPO0A PROTEIN

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Spo0A is the key protein in the initiation of sporulation in Bacillus. This response regulator contains two distinct domains, an N-terminal phosphoacceptor (or receiver) domain and a C-terminal DNA binding (or effector) domain. Recently it has been shown that the phosphoacceptor domain mediates dimerization of Spo0A on phosphorylation (3). A comparison of the crystal structures of phospho-

rylated and unphosphorylated N-Spo0A suggests that the structural changes accompanying activation extend from the active site region to the α4β5α5 surface of the protein (1,2). In particular the data suggest an important role in downstream signaling for Phe105, whose conformation alters dramatically upon phosphorylation, and its neighbour Tyr104 which is absolutely conserved in Spo0A from other spore-forming Bacillus and Clostridium species. In this study, we have prepared alanine substitutions of Tyr104 and Phe105 to probe their contribution to Spo0A function. Phosphorylation of the mutant proteins by phosphodonors such as phosphoramidate appears to be unaffected, but dimerization and DNA binding are abolished by the mutations. In addition, we have isolated several intragenic suppressor mutations to the Phe105Ala mutation, which were able to restore the function of Spo0A. We propose a model for the action of Spo0A in which the DNA binding activity of the effector domain is stimulated by phosphorylation and dimerization of the receiver domain.

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## A QUANTUM CHEMICAL STUDY OF THE STRUCTURE-NMR RELATIONSHIPS IN NUCLEIC ACIDS: SPIN-SPIN COUPLING & THE ORIENTATION ABOUT THE SUGAR-BASE BOND

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NMR vicinal spin-spin coupling constants $^3J_{C2/4\text{-}H1'}$ and $^3J_{C6/8\text{-}H1'}$ have been studied theoretically for deoxyribonucleosides as well as several modified bicyclo nucleosides as a function of the torsion angle chi. The structures of the nucleosides have been optimized at the DFT level of theory keeping the exocyclic backbone torsion angles frozen to their average experimental values and varying the glycosidic torsion angle chi. On the optimized structures, the DFT calculations of the spin-spin coupling constants have been performed using the combination of the FPT method (for the FC term) and the SOS_DFPT approach (for the PSO, DSO, and the SO terms) as implemented in the program deMon.[1].

Our calculations provide separate parameterizations of the Karplus equation for the vicinal C-H couplings for all of the deoxyribonucleosides studied. The results demonstrate that, contrary to approaches adopted earlier, different parameterizations are to be used for different nucleosides. For a given chi, the differences in the spin-spin coupling between different nuclosides reach up to 2 Hz. Vicinal spin-spin coupling across the glycosidic bond significantly differs for pyrimidines as compared to purines (in the syn region, the maximum of $^3J_{C6/8\text{-}H1'}$, is 4.0 for G but 5.8 for C). At the same time, also the particular purine/pyrimidine base matters (in the syn region, the maximum of $^3J_{C2/4\text{-}H1'}$, is 5.9 for C but 7.2 for T).

Another interesting result of our study is the observation of an "anti-Karplus" behavior of the vicinal coupling for some of the bases. The spin-spin coupling is thus more effective for the syn-periplanar than for the anti-periplanar arrangement of the coupled nuclei. This and other interesting features of the vicinal coupling mentioned above have been interpreted using the qualitative-MO approach together with the quantitative analysis of contributions to the coupling in the frames of the FPT-DFT approach. An interesting through-space contribution to the spin-spin coupling has been identified as the source of the anti-Karplus behavior for cytidine, as opposed to guanosine.

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## PROTEINS AND THEIR INTERACTIONS WITH SMALL MOLECULES STUDIED BY COMPUTER SIMULATIONS

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The presentation will introduce an on-going research project focused on proteins and their interactions with small molecules. Two systems are studied by computer simulations; (i) cyclin-dependent kinase-2 (cdk2) and its inhibition by purine-like compounds, and (ii) haloalkane dehalogenases.

Cyclin-dependent kinases are enzymes controlling the cell division cycle. A deregulation of cdk has been proved to occur in human primary tumors and in tumor cell lines [1]. It evoked potential application of cdk inhibitors in cancer therapy. Cdk2 regulates the G1/S cell phase transition

and DNA replication [2]. Cdk2 consists of two lobes [3]. An active site binding ATP or some purine-like inhibitors is positioned between the two lobes in the deep cleft. However, not all inhibitors from this category bind the enzyme in the same way as the ATP does. The project is focused on cdk2 and its complexes with purine-like inhibitors. The main goals are; (i) to understand how inhibitor changes protein behavior, (ii) to describe differences between binding modes, and (iii) to use results for rational drug design. (i) Molecular dynamic simulations are being used to study the differences between dynamic behavior of free cdk2 and its complexes with two inhibitors and ATP [4]. The X-ray structures of free cdk2 and its complexes with many ligands are known. (ii) Molecular docking is being used to study the binding of different purine-like inhibitors into the enzyme active site [5]. The MM-PB(GB)SA method and the energy decomposition analysis are being used to study binding modes of two inhibitors and ATP with cdk2. (iii) The results of molecular dynamics, molecular docking, energy decomposition analysis, and solvent behavior in the active site are being use for rational drug design.

Haloalkane dehalogenases are bacterial enzymes cleaving the carbon-halogen bond of the halogenated aliphatic compounds by a hydrolytic mechanism. These enzymes have a potential application in detoxification of subsurface pollutants and recovery of industrial side products. The project is focused on description (i) of differences between DhlA (*Xanthobacter autotrophicus*), LinB (*Sphingomonas paucimobilis*) and DhaA (*Rhodococcus sp.*) [6], (ii) monitoring substrates behavior in the active site [7,8]. (i) The essential dynamics is being use to obtain information about functionally relevant motions of three different haloalkane dehalogenases DhlA, LinB, and DhaA [9]. (ii) The formation of near attack conformation - conformation appropriate for reaction is being monitor to gain information about behavior of different substrates in the active site; 1,2-dichloroethane and 1-chlorohexane in the LinB, 1,2,3-trichloropropane in wtDhaA and mutDhaA.

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## STRUCTURE REFINEMENT OF NUCLEIC ACIDS USING VERY SMALL RESIDUAL DIPOLAR COUPLINGS

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Difficulties in structure determination of nucleic acids stem mostly from low proton density, lack of long-range restraints due to the elongated shape of the majority structures, and the small number of NOE restraints between the distant elements of secondary structure. Isotope labeling has improved the accuracy of local geometry determination and increased the number of NOE and torsion angle restraints. However, the lack of long-range restraints, for a long time, hindered the precise and accurate structure determination of nucleic acids. The recent introduction of liquid crystalline media, such as solutions of bicelles or filamentous phages, allowed measurements of residual dipolar couplings (RDC) even for molecules of small magnetic susceptibilities and provided therefore a direct way of obtaining the desired long-range and additional local restraints.

Number of one-bond $^{1}H$-$^{13}C$ and $^{1}H$-$^{15}N$ spin pairs in adenine, guanine, thymine, uracil, and cytosine bases, which supply sufficiently large residual dipolar couplings, is limited to just a few. In addition to one-bond RDCs also two-bond interactions may provide important source of restraints for structure refinement in nucleic acids. In purine and pyrimidine bases, a number of two-bond and one-bond homo- and heteronuclear distances has a well-defined length and can be successfully used during the structure calculations. As will be shown, a suite of spin-state-selective excitation (S3E) NMR experiments can be successfully employed for the measurements of small residual dipolar one-bond ($^{13}C$-$^{13}C$, $^{13}C$-$^{15}N$) and two-bond ($^{1}H$-$^{13}C$, $^{1}H$-$^{15}N$) coupling constants. Scalar and residual dipolar couplings were measured in the $^{13}C$, $^{15}N$-labeled DNA hair-

pin d(GCGAAGC) with very high precision and accuracy (1). Both "classical" NMR restraints and a set of residual dipolar couplings were used in the structure refinement of this DNA hairpin (2). The ensembles of 12 and 13 converged structures obtained using the simulated annealing molecular dynamics protocol with NOE and dihedral angles, and NOE, dihedral angles, and RDCs restraints, respectively, have an average of atomic RMSD (pairwise) of $0.39 \pm 0.15$ Å and $0.32 \pm 0.11$ Å. Both structures display the same conformational features and have been determined with very high precision as documented by the RMSD values. However, remarkable improvement in the definition of the A4 nucleotide, which sits at the top of the hairpin, has been obtained using the RDCs data. Position of this nucleotide is not well characterized by NOEs and just five small residual dipolar couplings helped to define structure of the A4 base with the precision close to that of other bases.

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## CHARACTERISATION OF BINDING SITES ON CD69 MOLECULE

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CD69 represents a very important activating molecule of the immunity system. It is presented on the surface of lymphocytes as a homodimeric receptor in the early phase of their activation. Each of monomers joined by disulphide bonds contains one lectin-like domain of C-type. Our previous study [1] revealed calcium-binding capacity and some carbohydrate ligands, but this question remained controversial [2].

In this study we try to characterize structure of this domain and describe its binding properties in more detail. From the results of binding experiments with $Ca^{2+}$ions we conclude that the carbohydrate recognition domain (CRD) of CD69 protein has a strong affinity to GlcNAc in a presence of these ions. This observation suggested the presence of binding site for calcium in this domain. The results of docking experiments based on the structure of CD69 molecule published recently showed us the hypothetical arrangement of that binding site. Amino acids proposed to be crucial for the binding of the $Ca^{2+}$ ion were mutated to alanin and the following binding experiments with radioactive $Ca^{2+}$ were accomplished. These results are in very good agreement with our predictions and thus provide a strong evidence for the role of calcium in this protein. We can conclude that CD69 is indeed a lectin with one calcium-binding site in the domain.

We were able to solve the question how many carbohydrate binding sites the protein actually has, and which their affinities to carbohydrate ligands are. Our experimental data are in good correlation with the results of computer modeling.

*This work was supported by grant MSM 113100001 from the Ministry of Education of the Czech Republic, grant 303/99/1382 from the Grant Agency of the Czech Republic and grant I-74679 from the Volkswagen Foundation.*

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## MODEL OF MODIFIED INTERNUCLEOTIDE LINKAGE FOR ANTISENSE USE: RAMAN STUDY OF G-$P_C$C(2'-5') NUCLEOTIDE SINGLE CRYSTAL

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In the search for efficient, universal strategies for curing viral and malignant diseases, much effort has been invested in the recent years in the development of novel approaches having as a target the very first events of the gene expression. Novel oligonucleotide analogues with modified internucleotide linkages, whose binding to the target sequence could prevent the production of pathogens, seem to be very promising. Investigation of physico-chemical properties of nucleotide analogues and their complexes with natural nucleic acid helps the impact of the internucleotide linkage modification on the properties of the respective nucleotides to be understood, and thus the efficiency of the search strategy to be improved.

The 5'-O-(guanosine-2'-O-phosphonomethyl)cytidine (G-$p_C$C(2'-5')) single crystal is the only crystallized structure [1] bearing one convenient modification, i.e., a phosphonate internucleotide linkage (Fig. 1). Polarized Raman spectra of the G-$p_C$C(2'-5') single crystal represent unique experimental data allowing force field parameters of the phosphonate linkage to be calculated. Considering the two possible geometries of G-$p_C$C(2'-5') detected in its crystal structure by X-ray diffraction, spectral characteristics of *g gt* and *tgt* phosphonate linkage conformation were acquired. Much information was withal obtained from

comparison of the crystal Raman spectra with those of powder sample and of aqueous solutions at various pHs, and also with published data concerning methylmethoxyphosphonate, a simple molecular model of this internucleotide linkage [2].

*This work is supported by the Grant Agency of CR (project 202/01/P102) and the Grant Agency of the Academy of Sciences of CR (A 4055902).*

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## DETEMINATION OF STRUCTURE, ENERGY, AND SPECTROSCOPIC PARAMETERS BY MEANS OF QUANTUM CHEMISTRY

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A presentation of the three projects running in parallel in the Department of Complex Molecular Systems:

i) Structure and Dynamic of Nucleic Acid (NA) base pairs and their microhydrated variants - ab initio calculation and computer simulation, Pavel Hobza

Chem. Rev. 1999,99,3247, 2000,100,143.

Potential and free energy surfaces of NA base pairs were studied using ab initio quantum chemical methods and molecular dynamic simulations. Both surfaces are characteristic by a large number of energy minima. Entropy is of the great importance in calculation of free energy surface of NA base pairs since its inclusion change the order of preferred structures. Potential energy surface of Adenine ... Thymine (AT) base pair shows that the most preferred structures are the base pairs which does not correspond to the base pairing situations found in DNA. The Watson - Crick type of base pairing was not found as the most stable structure neither in the study with N9 methylated A and N1 methylated T. JACS 2000,122,3495 JPC A 2001, 105, 1197.

ii) Interaction of DNA and RNA metal cations, Jiří Šponer

It is shown that the side specific interaction of metal cations ($Zn^{2+}$, $Mg^{2+}$, $Na^{+}$, $Cu^{+}$, cis and trans PtII) with DNA brings strong polarization and charge transfer effects which can be properely described only using the non empirical calculation methods. In the case of monovalent cations it is however possible to perform the molecular dynamic simulation with empirical potential. On the basis of theory - experiment (NMR, X-ray) comparison the direct influence of metal cation on proton transfer in DNA and base pair stability is presented. JPC B 104, 2000, 7535 JPC B 105, 2001, 12171 Inorganic Chemistry 40, 2001, 3269

iii) Use of NMR spin - spin coupling constants in structure determination - two examples, Vladimír Sychrovský

a) Calculated indirect NMR spin - spin coupling constants in the hairpin DNA molecule show the good owerall agreement with experiment and can be used for the base pair (AG versus GC) determination. The calculation of spin - spin coupling constants with and without

explicit water solvent molecules reveal the possibility of theory improvement by accounting for the solvent effects.

b) Mixture of the two conformers of Dipeptide Cyclo(L-Pro-L-Pro) considered in the study was detemined by fitting of their relative concetration ratio on experimental hydrogen - hydrogen NMR spin - spin coupling constants using the calculated coupling constants for each of the two conformer.

## INTERACTION OF DIFFERENT CYCLOSPORINES AND SILYMARINES WITH THE LIPIDIC PART OF HEPATOCYTE PLASMA MEMBRANE: THE ROLE OF THE SECOND AMINOACID SUBSTITUENT IN CYCLOSPORINES, DIFFERENT EFFECTS ON THE FLUIDITY OF MODEL AND NATIVE MEMBRANES.

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Cyclosporines (Cs) are non-ribosomatically synthesized cyclic undekapeptides that include some non-coded aminoacids. The most common of them is cyclosporine A (CsA) which is widely used in medicine as a powerful immunosuppressant in organ transplantations and for suppression of multidrug resistance in tumor chemotherapy. The cyclopeptidic structure features have some other important natural toxins (i.e. alga toxin microcystine, mushroom toxins amanitin and phalloidin etc.). Flavanolignans (called silymarines) from Milk Thistle (*Silybum marianum*) are known to help against hepatotoxic effects of

cyclic peptides. Mechanisms of actions in the liver cell are well known for both cyclosporines and silymarines. But much less is known about their transport through the cell plasma membrane. Because of their hydrophobic nature they are believed (at least partly) to enter the cell by passive diffusion through the lipidic part of hepatocyte plasma membrane.

We investigated the interaction of these compounds with different models of hepatocyte plasma membranes (from different phospholipid vesicles through plasma membrane vesicles up to hepatocytes). The interactions were monitored by changes in membrane lipid fluidity after cyclosporine or silymarine addition. The membrane fluidity was observed by measuring of the steady-state fluorescence anisotropy of diphenylhexatriene (DPH) and its polar derivative, TMA-DPH.

We found that for interactions of cyclosporines with lipids is the most important the side chain of the second aminoacid. The membrane fluidity increases in the raw: CsC < CsA < CsD < CsG, where the second aminoacid in CsC is threonin, in CsA: (α-aminobutyric acid, in CsD: valin and in CsG: norvalin. The same membrane fluidity as in case of CsA was found for interactions of cyclosporines with changes in other than second aminoacid; including CsH, which 3D structure completely differs from other cyclosporines (position 11 D-N-methylvalin instead of L-MeVal). The role of the second amino acid is supported by the fact that this side chain is in 3D structure of cyclosporines the most exposed residue to the space above the plane of cyclopeptide ring. On the other hand the change in amino acid number 1 which is the most exposed residue under the cyclopeptide chain, has no effect (i.e. fluidity measured for AcetylCsA was the same as for CsA). These results were found for each type of vesicles and for both probes. The changes were in order of $10^{-3}$ anisotropy units.

But different results were found in case of hepatocytes. Changes in membrane fluidity were found only in case of CsG but in the order of $10^{-2}$ anisotropy units. No changes were found in case of silymarines.

We concluded that:

1) The most important for hydrophobic interaction of cyclosporines is the second amino acid residue.

2) The intensity of the interaction increase with hydrophobicity of the second amino acid residue.

3) Hydrophobic interactions of cyclosporines in vivo can play a role only in case of CsG.

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## SIMULATION OF ABSORBANCE AND CIRCULAR DICHROISM SPECTRA OF PSII RC PIGMENTS, IMPLICATIONS FOR THE FUNCTION AND STRUCTURE

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Photosynthetic organisms are able to convert the energy of absorbed light into a chemical energy. One of the most important steps in this process is a charge separation, which occurs in a pigment-protein complex called reaction centre. The reaction centre of photosystem II (PSII RC) consists of five protein subunits bearing six molecules of chlorophyll *a*, two pheophytins *a* and two molecules of β-carotenoid. In general, electronic properties of pigment molecules in photosynthetic complexes are strongly affected by their interaction with the protein environment and with other pigments. As a consequence, the pigment molecules are able to perform multiple roles photosynthetic organisms. In antenna complexes they function in an absorption of light energy and its transfer, in reaction centres they are involved in charge separation as a primary electron donor and electron acceptor.

Our computational and experimental study is focused on implications of the RC PSII molecular structure for steady state absorption and circular dichroism spectra of this complex. For this reason it is especially important to know, beside the relative distances, mutual orientation of the pigment molecules. Unfortunately, the data so far obtained by X-Ray diffraction do not allow to extract the latter information with a sufficient accuracy. Therefore we used a combination of a modified theoretical model [4] (PDB code "1DOP") with addition of the peripheral chlorophyll No. 5 and 6 from the latest crystallography model. This approach led to be the best fit between experiment [3] and the spectra simulations. We have calculated the absorption and circular dichroism spectra in the point-dipole approximation [1] using dipole orientations as they can be extracted from PSII RC models and the X-Ray molecular structure data in *pdb* format of PSII RC [2] (PDB code "1FE1"). It is shown that the calculated spectra allow us to suggest the molecular origin of the light induced changes in the spectra observed experimentally [3].

The protein environment of the pigments was not involved in our calculation so far, but our preliminary results obtained by an application of experimental and theoretical molecular modeling methods support the correctness of the pigment molecules orientations in the theoretical model structure and call for the refinement of the existing X-Ray experimental PSII RC structure.

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## APPLICATION OF PROTEIN CRYSTALLOGRAPHY METHODS IN NATIONAL CENTER OF BIOMOLECULAR RESEARCH

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Proteins and nucleic acids perform many functions in living systems.The knowledge of their structure and functional properties allow understanding biochemical processes in organisms. Protein crystallography is widely used method for the structural determination of macromolecules at atomic resolution. The research of protein crystallography group of National Center for Biomolecular Research is focused on the structure solution of selected representatives of huge group of macromolecules.

Maize b-glucosidase, Zm-p60.1, has been suggested to be one of the key enzymes involved in regulation of plant development by releasing biologically active cytokinins from their storage and transport forms. The catalytically active form of Zm-p60.1 is a homodimer located in plastids.

The Zmp60.1 single crystals diffracting with synchrotron X-ray to at least 2.05 A have been prepared [1] and for structure determination the molecular replacement method has been successfully applied [2]. The enzyme is a/b hydrolase. The catalytic pair in the active centre is represented by two glutamic acids (Glu186 and Glu401), which were proposed to function as the acid/base and nucleophile, respectively.

The haloalkane dehalogenase from Sphingomonas paucimobilis UT26 (LinB) is the enzyme involved in the degradation of important environmental pollutant hexachlorocyclohexane. The enzyme hydrolyses broad range of halogenated cyclic and aliphatic compounds. There have been solved the 1.58 A crystal structure of LinB and 2.0 A structure of LinB with 1,3-propanediol, a product of debromination of 1,3-dibromopropane, in the active site of the enzyme [3]. Both structures have been solved by molecular replacement method. The enzyme is a/b hydrolase and contains a catalytic triad (Asp108, His272 and Glu132) in the lipase-like topological arrangement.

The bacterial alkalophilic serine proteases are characterised by their activity at alkaline pH and their broad substrate specificity. These enzymes share a common reactive centre composed of a catalytic triad and an identical fold consisting of two antiparallel ß-barrels. The pure alkaline serine protease from Nesterenconia sp. was used for crystallization experiment. The crystals belong to the space group R3 and there are 2 molecules per asymmetric unit. The determination of the 3D structure is pursued by multiple isomorphous replacement method.

The short fragment of DNA, d(GCGAAGC), creates extraordinarily stable minihairpin with high melting temperature Tm = 76 °C. The pink colored crystals have been used for data collection to 1.4 A resolution. The structure of the oligonucleotide is solved by single anomalous scattering (SAD).

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## STRUCTURE-FUNCTION RELATIONSHIPS IN A MAIZE β-GLUCOSIDASE ZM-P60.1

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A maize β-glucosidase encoded by *Zm-p60.1* cDNA cleaves the biologically inactive hormone conjugates cytokinin-O and N3-glucosides, releasing active cytokinins. In the roots of maize seedlings, Zm-p60.1 was localized to the meristematic cells and may function *in vivo* to supply the developing maize embryo with active cytokinin. To investigate structure-function relationships in Zm-p60.1, the enzyme was overexpressed in *Escherichia coli*, purified by a single-step metal chelate affinity chromatography and crystallized. The crystal structure of the wild type enzyme was solved at 2.05 A resolution al-

lowing molecular docking analysis to be conducted. This indicated that the enzyme specificity toward substrates with aryl aglycones is determined by aglycone aromatic system stacking with W373, and interactions with edges of F193, F200 and F461 located opposite W373 in a slot-like aglycone-binding site. Recently, these aglycone-active-site interactions were hypothesized to determine substrate specificity in inactive enzyme-substrate complexes of ZMGlu1, an allozyme of Zm-p60.1. Here we test this hypothesis by kinetic analysis of F193I/Y/W mutants. The decreased Km of all mutants confirmed the involvement of F193 in determining enzyme affinity towards substrates with an aromatic aglycone. Unexpectedly, a 30-fold decrease in $k_{cat}$ was found in F193I mutant compared to the wild type. Kinetic analysis and computer modeling demonstrated that the F193-aglycone-W373 interaction not only contributes to aglycone recognition as hypothesized previously but also co-determines catalytic rate by fixing the glucosidic bond in an orientation favorable for attack by the catalytic pair, E186 and E401. The catalytic pair, assigned initially by their location in the structure, was confirmed by kinetic analysis of E186D/Q and E401D/Q mutants. Unexpectedly, the E401D as well as C205S and C211S mutations dramatically impaired the assembly of a catalysis-competent homodimer suggesting novel links between the active site structure and dimer formation. Experiments designed to identify amino acid residues involved in discrimination between substrates at the level of aromatic ring substituents were initiated. Further, investigation of a link between fine tuning of formation of catalysis competent structure of the active site and dimerization-competent architecture of the monomer-monomer interface in the course of dimer assembly is in progress.

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