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## PREPARATION AND CRYSTALLIZATION OF AHP2 PROTEIN, THE SIGNAL TRANSMITTER FROM *ARABIDOPSIS THALIANA*

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Histidine phosphotransfer proteins (HPts) from *Arabidopsis thaliana* (AHP1-5) mediate signal transduction downstream from sensor histidine kinases (HK) to subsequent phospho-accepting response regulators (RR) via so called multistep phosphorelay (MSP). AHP proteins are involved in and potentially integrate various MSP signalling pathways (e.g. cytokinin, ethylene, osmosensing). However, our knowledge on structure of AHP proteins and its importance in the MSP signalling is scarce.

Here we report on expression, purification and preliminary crystallization of AHP2. Expression of AHP2 have been optimized and the sample quality was improved by using reducing conditions during purification procedure.

Crystallization conditions were found, but crystals diffracted only to 6Å resolution (X-ray source at Institute of Organic Chemistry and Biochemistry AS ČR, v. v. i. and X13 beamline at DESY, Hamburg). Further screening of optimal buffer conditions using differential scanning fluorimetry and limited proteolysis approach resulted in crystals diffracting to 3Å resolution (BESSY, Berlin). High-quality crystals for data collection are currently being produced.

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## BIOCHEMICAL CHARACTERIZATION OF DPCA FROM *PSYCHROBACTER CRYOHALOLENTIS* K5: THE FIRST EXTREMOPHILIC MEMBER OF THE HALOALKANE DEHALOGENASE FAMILY

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Haloalkane dehalogenases (HLDs, EC 3.8.1.5) are microbial enzymes which catalyze the hydrolysis of a carbon-halogen bond in halogenated aliphatic hydrocarbons, releasing a halide ion and a corresponding alcohol, as the reaction products. HLDs catalyze the reactions of great environmental and biotechnological significance with potential application in the bioremediation, the biosensing, the decontamination of warfare agents, the synthesis of optically pure compounds, the cellular imaging and the protein

tagging. The effective use of biocatalysts in these applications requires availability of enzymes with specific properties under harsh process conditions. Such properties can be easily obtained by isolation of novel biocatalysts from the extremophiles, producing the enzymes called extremozymes. These enzymes are able to operate under extreme conditions such as high or low temperature, high or low pH, pressure or high salinity.

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This study describes identification, isolation and biochemical characterization of a novel haloalkane dehalogenase DpcA from *Psychrobacter cryohalolentis* K5, representing the first extremophilic member of the haloalkane dehalogenase family. This enzyme was purified to homogeneity by metaloaffinity chromatography and biochemically characterized. Correct folding of DpcA was verified by the circular dichroism spectroscopy. Substrate specificity of the enzyme was tested towards thirty different halogenated compounds to confirm its dehalogenase activity. DpcA exhibited one of the narrowest substrate specificity profiles from all biochemically characterized HLDs. It was most active towards 1,3-dibromopropane, 1-bromohexane and 1-bromobutane, generally preferred

brominated and terminally substituted substrates. Compared to other HLDs, DpcA has the lowest thermostability ( $T_m = 34.7 \pm 0.6$  °C), possesses very unique temperature profile with maximal activity at 25 °C and retains more than 45 % of its activity below 15 °C. The psychrophilic properties of DpcA make this enzyme promising biocatalyst for various environmental applications, such as biosensing and/or bioremediation of groundwater contaminated by halogenated pollutants.

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## CHARYBDOTOXIN UNBINDING FROM THE MKV1.3 POTASSIUM CHANNEL: A COMBINED COMPUTATIONAL AND EXPERIMENTAL STUDY

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Charybdotoxin, belonging to the group of so-called scorpion toxins, is a short peptide able to block many voltage-gated potassium channels, such as mKv1.3, with high affinity. We use a reliable homology model based on the high-resolution crystal structure of the 94% sequence identical homolog Kv1.2 for charybdotoxin docking followed by molecular dynamics simulations to investigate the mechanism and energetics of unbinding, tracing the behavior of the channel protein and charybdotoxin during umbrella-sampling simulations as charybdotoxin is moved away from the binding site. The potential of mean force is constructed from the umbrella sampling simulations and combined with  $K_d$  and free energy values gained experimentally using the patch-clamp technique to study the free energy of binding at different ion concentrations and the mechanism of the charybdotoxin-mKv1.3 binding process. A possible charybdotoxin binding mechanism is deduced that includes an initial hydrophobic contact followed by stepwise electrostatic interactions and finally optimization of hydrogen-bonds and salt-bridges.

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A22

## UNIVERSAL STABILITY MODEL FOR GLOBULAR PROTEINS

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Amino acid substitution is the common approach to engineer stability and function of globular proteins. Stability prediction of protein mutants and decoys from their 3D structures requires universal and reliable energy function. Currently used functions for globular proteins are predominantly based on statistical force fields or on physics-based force fields lacking electrostatic interactions whose contribution vastly depends on the environment and solvation of

the denatured state and is therefore very difficult to be estimated solely from the native structure. Here we propose a simple model of globular protein stability based on all-atom force field calculations integrating all types of stability contributions. From the derived energy functions we are going to develop fast and reliable automatic online predictor of stability change upon mutagenesis from native structure.

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## ENGINEERING TUNNELS IN ENZYMES

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Enzymes are natural biocatalysts evolved for high selectivity and activity towards a wide range of substrates. Protein engineering makes a use of the knowledge gained from studies of protein structure/reactivity/selectivity relationships to construct improved biocatalysts for practical applications. Modifications of the residues forming the first or second shell of an active site are currently the most commonly used for successful design of constructs with a higher activity and selectivity, improved protein stability, or broadened substrate specificity. However, many enzymes show significant changes in their properties also due to mutations in the positions far from the active site [1]. Herein we present two examples of enzyme engineering focused on the residues forming the tunnels and their gates, as a new strategy for efficient control of enzyme properties.

DatA and LinB are examples of the haloalkane dehalogenases with buried active site. Their activity and selectivity reflects the properties of the substrate/product transport pathways. In this project, we have proposed mutations for the modification of the access pathways of these two enzymes using a novel approach. The rational design of mutants was assisted by the CAVER 3.0 software [2], which is the computational tools for the analysis of tunnels in static structures as well as in their ensembles from molecular dynamics simulations. The opening of the access tunnel in the DatA enzyme was shown to increase enzyme

activity, whereas the closing of the main access pathway in LinB enzyme decreased it significantly. Moreover, the designed mutants showed also highly altered substrate specificity. Our work provides evidence that the careful redesign of access pathways presents a powerful strategy for the precise control of the activity and selectivity of enzymes.

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## INTERMOLECULAR INTERACTIONS DISPLAYED BY X-RAY DIFFRACTION

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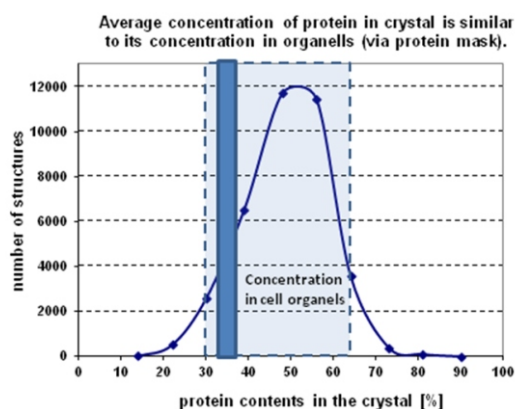
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X-ray diffraction methods have been developed in a user-friendly and relatively quick method for determination of macromolecular structure, for analysis of molecular dynamics and for determination of reaction paths taking place in these systems. In principle, the method has not any limitations as far as the complexity of the bio-macromolecular adducts. The results are fully based on experiment (no a priori assumptions on structure neither molecular modeling are required) and thus they are very reliable in comparison with any other method. Accuracy of atom positions depends always on quality of the local electron density, but for typical data measured at 1.8 Å resolution the expected accuracy is about 0.1 Å.

Protein crystal can be defined as a regular three-dimensional scaffold of protein molecules stabilized in solution by weak intermolecular forces. "The crystals" are in hydrodynamic equilibrium with solvent and must be measured in solution. Insensitive changes in solvent composition or solvent contents in the crystal quickly destroy diffraction quality of the sample.

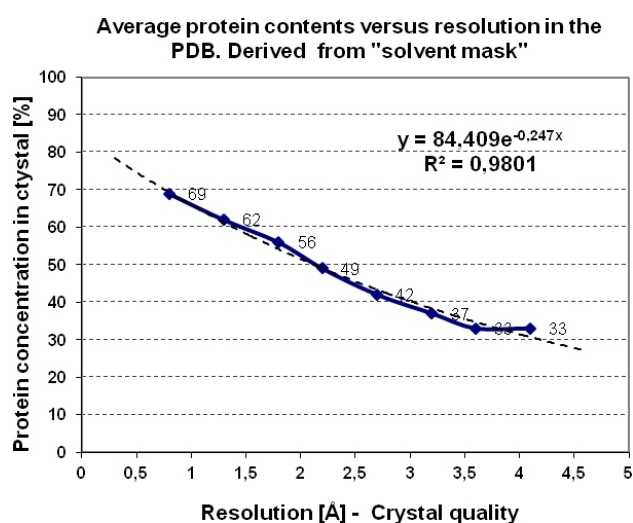
Protein crystals can exist in various concentrations (protein contents from 15 % to 75 %). However, for each molecular stacking, there is only a narrow range of solvent contents and the ingredient concentrations under which the stacking preserves its periodicity.



**Figure 1.** The graph shows a water contents in bio-macromolecular structures deposited in the PDB until 2012. It shows that a regular stacking of macromolecules into protein crystal is not possible when protein concentration in "the solid phase" is lower than 30 % or higher than 70 %. To compare it with a standard biological system: the dark rectangular area shows an average concentration of all biomolecules in cells and the lightly coloured area shows their expected concentrations in cell organelles.

X-ray structure analysis is a very reliable method and thus if one observes adhesion between macromolecules, it is a definite prove that this interaction exists under conditions present in the measured sample. However, because of large surface of macromolecules, each protein has several different interaction modes with preferences dependent on the solvent composition. Thus, the diffraction methods can show as a rule several complexes between two proteins with different affinity and stability. All the observed adhesion modes are real under given circumstances. The decision which adhesion mode dominates in the biological process in the cell may be simple or complex, but the preparation of several crystal forms showing all adhesion modes is desirable to get full view of the bio-processes in any case.

The real adhesion mode dominating in the bioprocess under investigation can be different from the adhesion modes observed in solution because of many competitive intermolecular interactions and spatial restrictions in the living cell. Even more, the biologically relevant adhesion mode need not be the most stable in solution, because the proteins are often oriented by anchoring in membrane or are simply temporary complexed with other molecules forming the respective organelle of the cell. Thus, many unknown factors can change the adhesion properties of the inspected macromolecule in the bioprocess under investigation.



**Figure 2.** A higher content of water in the measured protein crystal leads on average to numerically higher "resolution" (to lower amount of diffraction data). The "protein surface modifying agents PSMA" can help to stabilize the macromolecular stacking in regular lattice and to get the resolution under the desirable limit 1.8 Å corresponding roughly to the accuracy of atom positions about 0.1 Å on average [3, 4].

X-ray structure analysis is optimal tool for inspection of these phenomena, because the crystalline samples studied have very similar concentration of the protein material as it is in organelles of the living cell (see Figure 1). The talk explains how “protein surface modifying agents PSMA” [1, 2] allow growing different crystal forms showing different adhesion modes between proteins to get as far as possible a complete review of all adhesion modes of the protein studied. It also explains a role of PSMA in increasing the accuracy of the experimentally determined atom positions under the desirable limit 0.1 Å (see Figure 2). Applications of polymer materials as “protein surface modifying agents” and behaviour of hydrophilic polymers in the bio-environment are summarized in [3]. The practical usage of these considerations in development of new crystallization screens were published in [4]. It is expected, that the new

more complex usage of the X-ray diffraction methods as described here can provide more realistic insight on the real behaviour of molecules in their natural bio-environment.

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## PHOTOSYNTHETIC PSBR PROTEIN FROM HIGHER PLANTS

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Photosynthesis is a chemical process that converts carbon dioxide into organic compounds, especially sugars, using the energy from sunlight [1]. Photosynthetic organisms are called photoautotrophs, since they can create their own food. In plants, algae, and cyanobacteria photosynthesis uses carbon dioxide and water, releasing oxygen as a waste product. Photosynthesis is vital for all aerobic life on the Earth. In addition to maintaining normal levels of oxygen in the atmosphere, photosynthesis is the source of energy for nearly all life on the Earth, either directly, through primary production, or indirectly, as the ultimate source of the energy [2].

Here we report identification of the primary structure of PsbR protein from *Pisum sativum L.* Primary and secondary structure of higher plants PsbR protein has not been discovered yet, because this protein is highly unstable outside of its normal occurrence (Photosystem II complex). The PsbR protein likes many others, which are denoted PsbA – PsbZ, plays an essential role in photosynthesis of higher plants. It is a part of oxygen-evolving complex (OEC) and therefore it is directly contributing to production of oxygen.

First of all we extracted RNA from *Pisum sativum L.* With cDNA (from mRNA) we were capable to use it as template for the PCR reaction, where we were working with our designed degenerative primers, because as mentioned above, a complete structure of the protein has not

been solved up to date. With comparison of degenerative and non-degenerative primers we were able to obtain PCR product of PsbR protein from pea that was transformed into pBluescript vector and get sequenced. To find out sequence of mature peptide the MALDI TOFF spectroscopy was applied.

Simultaneously we produced *psbR gene* from mRNA isolated from *Spinacia oleracea*. Primary structure of spinach PsbR protein is known and we prepared expression vector with its anchor as HisPsbR and without anchor as PsbR. The purification conditions were optimized to get stable proteins that will be used for other structural analysis.

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## SPR STUDIES ON THE INTERACTIONS OF TRP CHANNELS AND THEIR ACTIVITY MODULATORS

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Transient receptor potential (TRP) channels consist of a diverse group of cation channels that contains more than 30 members. The channels participate in many sensory and physiological processes. The vanilloid receptor (TRPV1) is one of the best characterized members of the TRPV subfamily. This nonselective cation channel serves as a polymodal receptor for various potentially harmful signals. Activation is caused by diverse stimuli, such as noxious heat (>43 °C), low pH (<5.4) and chemicals such as capsaicin. TRPM3 is one of the least investigated proteins of the TRP family of ion channels. It was described to be involved in Ca<sup>2+</sup> homeostasis in kidney cells and to be activated by noxious heat. Intracellular termini of TRP channels are involved in regulation of their activity via binding of intracellular ligands as CaM or phosphatidylinositol – 4,5 - biphosphate (PIP2). In order to characterize complex forming we used surface plasmon resonance (SPR) as a suitable tool. Employing two different types of SPR protocols, we have explored interactions of C-terminal regions of TRPV1 with phospholipids namely L777-S820 and two

novel binding sites of TRPM3 for CaM namely A35-K124 and H291-G382. All SPR measurements assessing the PIP2 and protein equilibrium dissociation constant were performed at 25 °C using a liposome-coated NLC chip in ProteOn XPR36 Protein Interaction Array System (Bio-rad, Hercules, CA, USA). All SPR measurements characterizing TRPM3 / CaM interactions were performed on CaM coated GLC chip on ProteOn XPR36 (Bio-Rad, Hercules, CA, USA). TRPV1 showed relatively high affinity and selectivity toward PIP2, KD 1.88 +/- 0.46 μM. TRPM3 constructs A35-K124 and H291-G382 bound CaM with high affinity, KD 0.198 +/- 0.018 and 0.481 +/- 0.074 μM respectively. Site directed mutagenesis experiments further revealed that basic residues within these binding sites may play a crucial role in TRPV1 and TRPM3 channels binding to PIP2 and to CaM respectively.

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## PROTEIN OLIGOMERIZATION IN ALEURIA AURANTIA LECTIN FAMILY – IMPORTANCE AND DIFFICULTIES

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The formation of quaternary structure is crucial for function of many different proteins. Many of them are fully functional only upon oligomerization or change their properties due to binding of several subunits to each other. The determination of protein oligomeric state is important to fully describe its structural and functional properties. Combination of several methods like size-exclusion chromatography, dynamic light scattering or analytical ultracentrifugation together with X-ray crystallography enable us to see deeper into protein complexity.

Oligomerization and multivalency are very important for carbohydrate binding proteins, lectins, responsible among others for pathogen binding to host tissues. In some

cases the binding sites are formed in between two subunits. In the *Aleuria aurantia* lectin family, oligomerization plays role in two different ways. Whereas bacterial lectins like RSL from *Ralstonia solanacearum* [1] or BambL from *Burkholderia ambifaria* [2] form trimers to establish complete b-propeller fold, eucaryotic lectins like AAL from *Aleuria aurantia* [3] or AFL from *Aspergillus fumigatus* combine two -propellers to further increase avidity effect. In addition, although the tertiary structures of these proteins are very similar, there are interesting differences in dimerization. Detail knowledge in this area can help us to design highly efficient artificial lectins, as well as improve

the treatment strategy in cases, where lectins play integral role in infection process.

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## UTILIZATION OF MOLECULAR DYNAMIC METHODS FOR IDENTIFICATION OF STABLE CYP1A2-CYTOCHROME B<sub>5</sub> BINARY COMPLEXES

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Cytochrome P450 (CYP) represents a large group of enzymes oxidizing drugs and chemical carcinogens. Eukaryotic CYPs interact with other membrane proteins located in the endoplasmic reticulum. Cytochrome b<sub>5</sub> (cyt b<sub>5</sub>), which is also present in endoplasmic reticulum (ER), may enhance, inhibit or have no effect on enzymatic activity of CYPs, depending on the particular CYP isoform and the substrate. Hence, the cyt b<sub>5</sub> has the potential to significantly modulate CYP mediated metabolism of xenobiotics. The cyt b<sub>5</sub>-mediated modulation of CYP activity is attributed to the formation of specific protein-protein interaction. Exact 3D structure of the binary complex has not been experimentally determined. However, indirect data based on mutagenesis studies and chemical cross-links indicate that the convex and acidic surface of cyt b<sub>5</sub> binds to the basic concave surface of a CYP.

Presented study is focused on prediction of the CYP1A2-cyt b<sub>5</sub> binary complex structure, using contemporary methods of theoretical chemistry. In the first step,

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flexible protein-protein docking method implemented in HADDOCK software was employed to obtain a set of plausible orientations of soluble domains of both cytochromes. Further several individuals were selected according to rough estimations of binding energies and mutual orientations of both protein structures. These binary complexes were subjected to stability evaluation using classical molecular dynamic method implemented in NAMD software. Further we employed steered molecular dynamic protocol. This method was used in order to compare binding free energies of individual CYP1A2-cyt b<sub>5</sub> complexes obtained by protein-protein docking.

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## STRUCTURAL BASIS OF THE 14-3-3 PROTEIN-DEPENDENT REGULATION OF FOSDUCIN

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Phosducin (Pdc), a regulatory and highly conserved phosphoprotein, plays an important role in the regulation of G protein signaling by modulating the amount of G<sub>t</sub> heterotrimer through the competition with the G<sub>t</sub> subunit for binding to the G<sub>t</sub> complex [1]. Besides its well-established role in the regulation of G protein signal-

ing, Pdc is also involved in the transcriptional control and the modulation of blood pressure. The function of Pdc is regulated through its phosphorylation and the binding to the regulatory 14-3-3 protein [2]. The 14-3-3 proteins are scaffolding molecules that regulate the function of other proteins through a number of different mechanisms. The exact role of the 14-3-3 protein in the regulation of Pdc is,



however, still elusive. In this work, we performed a biophysical analysis of the Pdc:14-3-3 complex. First, the analytical ultracentrifugation was used to study the binding affinity and the stoichiometry of the complex. Our data show that Pdc phosphorylated simultaneously at Ser54 and Ser73 form a stable complex with the 14-3-3 protein while unphosphorylated or singly phosphorylated forms of Pdc show no significant binding to 14-3-3. The time-resolved emission spectroscopy of tryptophan and dansyl fluorescence was then used to probe the structural changes of Pdc molecule induced by both the phosphorylation by itself and the 14-3-3 protein binding. The time-resolved tryptophan fluorescence intensity and anisotropy decay measurements show significant structural alterations in the region surrounding Trp29 at the N-terminus of Pdc molecule upon the 14-3-3 protein binding. The time-resolved fluorescence measurements of Pdc labeled at five different

positions by 5-(((acetylamino)ethyl)amino) naphthalene-1-sulfonic acid (AEDANS) revealed that the 14-3-3 binding induces significant structural changes in both the N- and C-terminal domains of Pdc. In addition, our data also indicates that the simultaneous phosphorylation at both Ser54 and Ser73 by itself significantly affects the structure of Pdc molecule.

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A30

## STRUCTURE-BASED DISCOVERY OF FLOWERING HORMONE RECEPTOR

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Florigen, a mobile floral induction factor encoded by FLOWERING LOCUS T (FT) and its homologs, activates floral identity genes such as APETALA1 (AP1) at the shoot apex. Florigen is produced in leaves and is transmitted through the phloem to the shoot apex, where it induces flowering. A number of recent reports have provided evidence that *Arabidopsis* FT protein (Hd3a in rice) is a key component of florigen. In the shoot apical meristem, FT activates AP1 transcription and induces flowering by interacting with the bZIP transcription factor FD, although the detail of the interaction between FT and FD has not yet been clarified. In rice, the closest homolog of FD, OsFD1, has been identified based on its function and homology with maize DLF1. The FT-FD interaction is required for flowering, and phosphorylation of residue T282 in the C-terminal region of FD may be critical for this interaction and floral initiation. Here we found the rice florigen Hd3a directly interacts with 14-3-3 proteins, but not with a tran-

scription factor OsFD1. These 14-3-3 proteins play a key role by mediating an indirect interaction between Hd3a and OsFD1. Therefore we have determined the 2.4-Å crystal structure of a rice floral induction complex, named the florigen activation complex (FAC), which consists of Hd3a, OsFD1, and 14-3-3 protein. This FAC structure clearly explains the activation mechanism of the floral identity genes by florigen. Finally, functional studies using rice cultured cells and transgenic rice plants proved that the 14-3-3 proteins function as intracellular receptors of florigen to activate floral identity genes [1].

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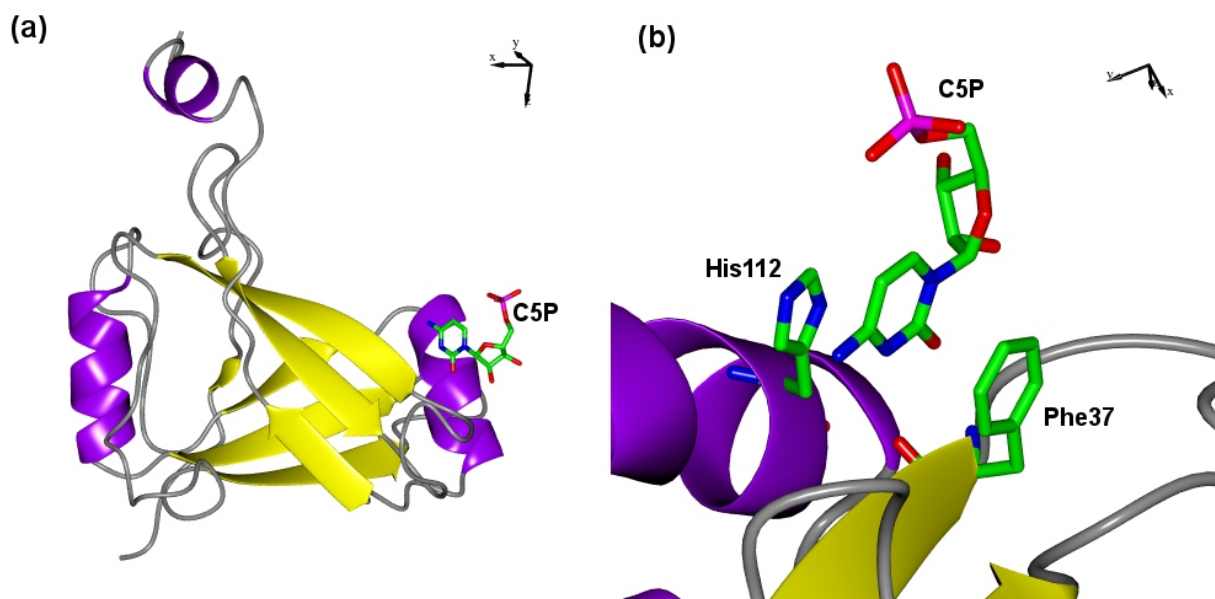
A31

**BDV MATRIX PROTEIN: FROM MUTATION TO FUNCTION****P. Kolenko<sup>1</sup>, P. Dautel<sup>1</sup>, R. Novotny<sup>2</sup>, A. Martin<sup>2</sup>, C. Parthier<sup>1</sup>, M. Schwemmler<sup>2</sup>, M.T. Stubbs<sup>1</sup>**<sup>1</sup>*Institut für Biochemie und Biotechnologie, Martin-Luther Universität, Kurt-Mothes-Straße 3, 06 120 Halle (Saale), Germany*<sup>2</sup>*Institut für Virologie, Universität Freiburg, Hermann-Herder-Straße 11, 79 104 Freiburg, Germany  
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Borna disease virus (BDV) is a neurotropic virus that typically infects horses, sheep and other farm animals [1]. Recent studies have also shown that genomic BDV-like elements were inserted into the mammalian genome, including humans [2]. Besides Ebola, Marburg and Vesicular stomatitis virus, BDV belongs to the order of Mononegavirales [3]. One of the six proteins encoded by the BDV genome (negative stranded non-segmented RNA) is the matrix (M) protein [4]. BDVM occurs dominantly in form of a homo-tetramer with a total molecular weight of 65 kDa. Monomers of BDVM fold into an L-shaped  $\alpha$ -sandwich consisting of 6 antiparallel strands that are surrounded by several  $\alpha$ -helices (see Fig. 1). BDVM binds short fragments of RNA and is able to protect them from degradation. Despite a wide range of studies, the role of BDVM in life cycle of the virus is not fully understood, yet. We have designed several mutant variants of the BDVM protein, and analyzed them using analytical size-exclusion chromatography, analytical ultracentrifugation, RNA-PAGE, spectrometric methods, and X-ray crystallography. Finally, we performed pairwise comparison of the mutant variants with the wild type BDVM. We have observed novel oligomeric states, and also secondary RNA binding sites. According to our experiments, both aspects play a role in growth of the virus.

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**Figure 1.** Overall structure of BDVM (a), and its nucleotide binding site (b). BDVM is represented by secondary structure elements, cytidine-5'-monophosphate and interacting residues His112, and Phe37 are represented by sticks. The figure was generated using CCP4MG [5].



A32

## MAIZE ALDEHYDE DEHYDROGENASES FROM THE FAMILY 7 AND 10

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Aldehyde dehydrogenases (ALDHs) comprise a protein superfamily of NAD(P)<sup>+</sup>-dependent enzymes (EC 1.2.1). They have been considered as general detoxifying enzymes, which eliminate biogenic and xenobiotic aldehydes to the corresponding carboxylic acids. Up to date, twelve ALDH families have been described in plants, but only a small number of the enzymes have been functionally characterized despite the existence of a large number of coding genes. In this work, we analyzed several ALDHs from maize (*Zea mays*) belonging to the families 2, 3, 7 and 10 with a special interest in the latter two families. For ALDH7, there are also synonymous terms <sup>1</sup>-piperidine-6-carboxylate dehydrogenase or <sup>1</sup>-aminoadipic semialdehyde dehydrogenase used. ALDH7 is mainly connected with lysine metabolism as it catalyzes the conversion of <sup>1</sup>-aminoadipic semialdehyde (-AASA) to <sup>1</sup>-aminoadipate. ALDH10 is an aminoaldehyde dehydrogenase (AMADH, EC.1.2.1.19), often reported as a betaine aldehyde dehydrogenase (BADH, E.C. 1.2.1.8), and oxidizes <sup>1</sup>-aminoaldehydes to the corresponding amino acids such as <sup>1</sup>-alanine, <sup>1</sup>-aminobutyric acid, <sup>1</sup>-butyrobetaine or glycine betaine. The enzyme is connected with the meta-

bolic pathways of polyamines, arginine, lysine and choline. Selected genes coding for ALDHs were cloned and expressed in T7 *E. coli* cells. The recombinant enzymes were thoroughly characterized and their identity was verified by MALDI-TOF peptide mass fingerprinting. Maize ALDH7 utilizes NAD<sup>+</sup> but not NADP<sup>+</sup> as a coenzyme and prefers <sup>1</sup>-AASA to other aliphatic and aromatic aldehydes. Three maize ALDH10 members (AMADH1a, 1b and 2) preferentially oxidize 3-aminopropionaldehyde, 4-aminobutyraldehyde and 4-guanidinobutyraldehyde (but not <sup>1</sup>-AASA) and they use NAD<sup>+</sup> as a coenzyme. Our results confirm a previous suggestion that the presence of two highly unconserved Trp residues at the substrate channel results in higher affinity to <sup>1</sup>-aminoaldehydes. Representatives of both families were crystallized. So far, the crystal structure of ZmAMADH1 has been solved up to 1.95 Å resolution.

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A33

## PREPARATION OF THE REGULATORY DOMAIN OF TYROSINE HYDROXYLASE FOR NMR STUDIES

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Tyrosine hydroxylase belongs to the group of hydroxylases of aromatic acids, class oxydoreductases and subgroup oxygenases. This enzyme catalyses key step in the biosynthesis of catecholamine neurotransmitters – the conversion of the tyrosine to the 3,4-dihydroxyphenylalanine. We can find it mainly in cells of the adrenal gland, in the heart, in the liver, in gonads and in the central nervous system [1, 2].

The tyrosine hydroxylase has the homotetrameric structure and contains three diverse structural domains: N-terminal regulatory domain, catalytic domain and C-terminal tetramerization domain [3]. The activity of tyrosine hy-

droxylase is regulated by phosphorylation and through the regulation of its expression. All phosphorylation sites (Ser-8, Ser-19, Ser-31 and Ser-40) are located within the regulatory domain [2]. Phosphorylation at Ser-40 by cyclic AMP-dependent protein kinase (PKA) induces the most potent activation of tyrosine hydroxylase. It has been proposed that phosphorylation of Ser-40 alters the conformation of the regulatory domain and its interaction with the catalytic domain. Phosphorylation at Ser-19 induces binding of the 14-3-3 protein, which affects the structure of the regulatory domain and protects it against dephospho-

rylation (at phosphorylated Ser-40) and its degradation [4, 5].

Since the structure of the regulatory domain is still unknown we decided to perform its structural characterization using NMR techniques. The regulatory domain of tyrosine hydroxylase was expressed as six-His-tag fusion protein by IPTG induction for 12 h at 20 °C and purified from *Escherichia coli* BL21(DE3). Its purification consists of two steps: the chelating chromatography and the size-exclusion chromatography on Superdex 200 column. The dynamic light scattering, the 1H and HSQC spectra were used to verify that the recombinant protein is not aggregated and can be used for further experiments.

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A34

## STRUCTURE-FUNCTION STUDY ON PLANT S-NITROSOGLUTATHIONE REDUCTASE FROM TOMATO

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S-nitrosogluthathione reductase (GSNOR), also known as S-(hydroxymethyl)glutathione (HMGS) dehydrogenase (EC 1.1.1.284), belongs to the large alcohol dehydrogenase (ADH) superfamily, namely to the ADH3 family. GSNOR catalyzes the oxidation of HMGS to S-formylglutathione using NAD<sup>+</sup> as a coenzyme. However, the enzyme catalyzes also the NADH-dependent reduction of S-nitrosogluthathione (GSNO). In general, GSNO serves as a nitrogen oxide (NO) donor in distant tissues and in humans the alternations in the metabolism of GSNO are linked to several lung diseases including asthma. NO and NO-related molecules such as S-nitrosothiols (S-NOs) play a central role in the regulation of normal plant physiological processes and host defense. The enzyme thus participates in controlling the cellular homeostasis of S-NOs and in the metabolism of reactive nitrogen species. Although GSNOR has been recently characterized from several organisms, this study represents the first detailed biochemical and structural characterization of a plant GSNOR from tomato (*Solanum lycopersicum*). The crystal structures of the apoenzyme and the enzyme in complexes with NAD<sup>+</sup> and NADH with GSH were solved up to 1.9 Å resolution. They represent the first structures within the plant ADH3 family.

The crystals were primitive orthorhombic, space group P2<sub>1</sub>2<sub>1</sub>2 and P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, and contained one dimer per asymmetric unit. The calculated Matthews coefficient was ~ 2.4 Å<sup>3</sup> Da<sup>-1</sup>, corresponding to a solvent content of 49 %. Each monomer comprises two zinc atoms and consists of a catalytic and a coenzyme domain. The solved structures confirm that coenzyme binding is associated with a zinc movement and changes in its coordination. The first zinc atom functions as a Lewis acid and activates alcohols or other substrates during catalysis while the second one has a structural role. The enzyme preferentially oxidizes HMGS although some other alcohols such as cinnamylalcohol, geraniol and 12-hydroxydodecanoic acid are also good substrates. In the reductase mode with NADH the enzyme exhibits a high affinity and catalytic efficiency for GSNO while glutathione and S-methylglutathione behave as non-competitive inhibitors.

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## PREPARATION OF THE 14-3-3:ASK1 KINASE COMPLEX FOR STRUCTURAL STUDIES

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ASK1 (Apoptosis signal-regulating kinase, MAP3K5) plays a critical role in the regulation of the apoptosis triggered by cellular oxidative stress, immune response or anticancer drugs as well as the development of several diseases [1]. ASK1 is a serine/threonine protein kinase from MAP3K family involved in the activation of p38 and JNK signal pathways by direct phosphorylation of MEKK4 and MEKK6. Its activity is strictly controlled by diverse mechanisms such as phosphorylation, oligomerization and protein-protein interactions. The 14-3-3 protein has been identified as one of the most important regulators of ASK1 enzymatic activity [2]. It binds to phosphoserine 967 and suppresses the enzymatic activity, and thus the signaling potential. Molecular mechanism of this regulatory interaction is still unknown. Here, we report the preparation and basic biophysical characterization of the 14-3-3:ASK1 complex for subsequent structural studies to understand and explain this interaction. We optimized expression, pu-

rification and phosphorylation protocols for the preparation of human recombinant ASK1 catalytic domain. Purification protocol for the preparation of the 14-3-3 protein had been developed previously. The result of ASK1 phosphorylation at Ser967 *in vitro* was verified by MALDI-TOF mass spectrometry. Interactions between ASK1 and the 14-3-3 protein were further studied using native electrophoresis and analytical ultracentrifugation.

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A36

## ENGINEERING ENZYME RESISTANCE TO ORGANIC CO-SOLVENT

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Haloalkane dehalogenases (EC 3.8.1.5) are representatives of the  $\alpha$ -hydrolase fold superfamily [1]. These enzymes can hydrolytically cleave a halogen atom from more than hundred chlorinated, brominated or iodinated alkanes and their derivatives [2-3]. Access of halogenated substrates to the buried active site, and egress of formed alcohols and halides are facilitated by the protein tunnels [4]. The industrial use of these enzymes is limited by their instability in the presence of co-solvents, which are used for solubilization of hydrophobic substrates. Here we present a systematic protein engineering study, which examines features determining resistance of haloalkane dehalogenases towards organic co-solvent dimethyl sulfoxide. The initial random mutagenesis of a dehalogenase gene followed by a colorimetric screening in 42% dimethyl sulf-

oxide revealed importance of the residues lining the access tunnels for protein stability. Substitutions introduced to the residues lining the main access tunnel led to the enzyme variants with improved kinetic stability, increased structural resistance towards dimethyl sulfoxide and elevated structural thermostability. The experimental results were supported by molecular dynamics simulations. Based on our findings, the resistance of haloalkane dehalogenases and possibly also other enzymes with buried active sites can be improved by modification of their access tunnels.

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A37

## REACTION MECHANISM PRINCIPLES OF THE BIFUNCTIONAL PLANT NUCLEASE TBN1

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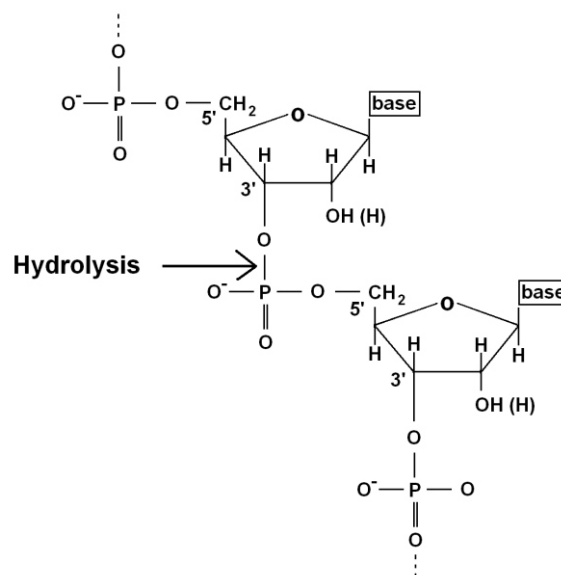
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Bifunctional nuclease TBN1 (UniProt sequence accession no. Q0KQV0) from *Solanum lycopersicum* (tomato) is a Zn<sup>2+</sup>-dependent plant glycoprotein composed of 277 amino acids with a molecular mass of 31.6 kDa (about 37 kDa when glycosylated). TBN1 belongs to plant nuclease I family and plays an important role in specific apoptotic functions, vascular system development, stress response and tissue differentiation in plants [1]. In addition, TBN1 exhibits anticarcinogenic properties [2]. Therefore, a detailed structural study of this enzyme can contribute to development of new drugs for cancer, bacterial and viral disease treatment. Nuclease P1 from *Penicillium citrinum* with 24% sequence identity, the structure of which is known (PDB ID 1ak0) [3], is probably the closest structural homologue of TBN1.

Heterologous expression of TBN1 in tobacco leaves yields amounts and quality of the enzyme suitable for structural studies. Crystals with sufficient quality for X-ray diffraction analysis can be obtained. The first diffraction experiments were performed using an in house Gemini Enhanced Ultra diffractometer with the Atlas CCD detector (Oxford Diffraction) and three different crystal morphologies were identified (orthorhombic, rhombohedral and trigonal). Datasets for structural analysis were collected at the synchrotron radiation source BESSY II (Helmholtz-Zentrum Berlin), beam line MX-14-1, with a MARmosaic CCD detector. Presence of zinc in the protein was confirmed by X-ray fluorescence and an absorption edge scan and two MAD datasets (for a rhombohedral and a trigonal crystal) were collected. The phase problem was solved using the SHELXC, D and E program suite [4]. A cluster of zinc ions was identified and a preliminary structure model was built by placing the P1 nuclease structure into the experimental electron density using MOLREP [9]. Building and refinement of this structure was limited [6, 7]. The trigonal crystal diffracted to 2.15 Å resolution and the final



**Figure 1.** TBN1 cleaves the bond between the 3' hydroxyl group and phosphate in nucleic acids by hydrolysis.

model was built and refined using this data. The TBN1 structure is mainly  $\alpha$ -helical with three Zn ions placed in the active site at the bottom of the wide groove. Three oligosaccharides bonded on the surface serve primarily as a shielding of the hydrophobic regions and therefore contribute to solubility of the enzyme. The main differences between TBN1 and P1 are in the composition of the side chains around the active site, in the glycosylation pattern and also in the shape and in the electrostatic potential on the surface.

TBN1 acts as phosphodiesterase cleaving the bond between phosphorus and 3' hydroxyl group in both single



stranded and double stranded forms of DNA and RNA (Figure 1). TBN1 prefers bonds adjacent to adenine, generates 5'-mononucleotides as end products and also shows 3' nucleotidase activity. Trinuclear  $Zn^{2+}$  cluster coordinated at the bottom of the active site cleft is crucial for the activity. Hydrolysis of the phosphodiester bond is caused by a nucleophilic attack of the activated water (hydroxide) molecule followed by creation of pentacoordinated transition state and its breakup into the products.

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## CHARGE TRANSPORT IN DNA OLIGONUCLEOTIDES WITH VARIOUS BASE-PAIRING PATTERNS

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Charge migration along DNA double-helical molecules may be of biological importance as the extended electronic states of DNA could play a role in the processes of sensing and/or repair of DNA damage. We have combined various experiments and theoretical models to elucidate further the DNA charge transfer process in terms of DNA base pairing patterns, base stacking interaction and the role of the sugar-phosphate backbone. Through an exploration of the relation between the physical and chemical statuses of a particular DNA modification and its charge transport properties, we intended to affect the extremely complex charge transport process and its biological and technical signifi-

cance. Our experimental data as well as the results from the theoretical models and calculations indicate that DNA charge transport strongly depends on the presence of various perturbations, in particular irregularities in base-pairing, base-stacking patterns and various metals/metal groups presence/interactions. Even seemingly unimportant structural perturbations caused by the presence of mismatched base-pairs affect conductivity to a greater extent than expected from conformational changes and decreased thermal stability alone.

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## PREPARATION AND STRUCTURE DETERMINATION OF T41I/T78I MUTANT OF THE M-PMV MATRIX PROTEIN

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Mason-Pfizer monkey virus (M-PMV) belongs to betaretroviruses and is widely used as a model organism for studies of the late phase of the life cycle of retroviruses. Matrix protein which is naturally modified by the rest of the myristic acid on the N-terminus, plays key role in the transportation of the immature retroviral particles to budding sites and is responsible for an interaction with the plasma membrane. Mutation T41I/T78I in the matrix protein causes either a change in the hydrophobicity of the cavity in which the myristoyl is nested or a change in an in-

teraction site of the matrix protein with the plasma membrane. The outcome is that the immature viral particles can't bud from the infected cell.

Our work focused on the preparation of the myristoylated T41I/T78I mutant of the matrix protein of M-PMV. The mutant protein was analyzed by the NMR spectroscopy and the 3D structure was determined. The liposome binding assay was used to study an interaction with the plasma membrane.

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## BEHAVIOR OF WATER, IONS AND SMALL ORGANIC MOLECULES NEAR QUARTZ (101) SURFACES

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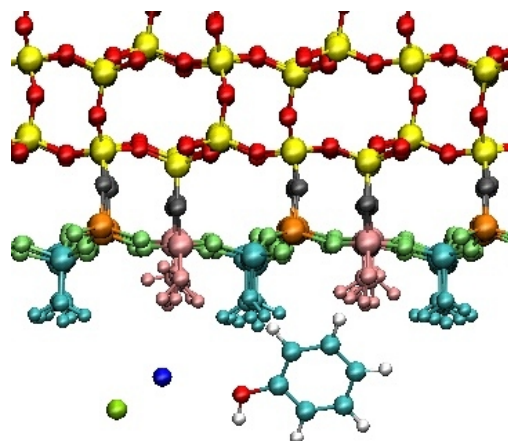
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We focused our attention on molecular dynamics simulations of the interactions of quartz surfaces with aqueous solutions of ions and small organic molecules representing functional groups of organic matter.

Simulated quartz surfaces covered the range of surface charge densities 0.00, -0.03, -0.06 and -0.12 C/m<sup>2</sup>, approximately corresponding to pH values 4.5, 8.5, 9.5 and 10.

We found increasing water ordering within two water layers closest to the surface with increasing surface charge density. Also sodium ions adsorption increase with increasing charge density and specific adsorption pattern can be found between negatively charged Si-O<sup>-</sup> species and sodium cations.

Humic acids and humates have heterogeneous and complex structures with different functional groups including acidic, hydrophilic, and hydrophobic groups. Humic acids are able to interact with both organic and inorganic substances such as nutrients, metals, hydrophobic organic compounds and mineral surfaces. Understanding their structure and interactions can give us important information about their degradability, toxicity, and transport properties. Also they are one of the major causes of so-called 'bio-fouling' of nanofiltration and reverse osmosis membranes which are used in industry for water purification, water desalination and wastewater treatment.



Adsorption of small organic molecules representing basic building blocks of larger biomolecules and organic matter is also subject of this study. As model molecules, benzoic acid, phenol, and salicylic acid were chosen.

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