

5th Discussions in Structural Molecular Biology

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Conference Committee

Bohdan Schneider, Ivana Kutá Smatanová, Radomír Kužel, Dalibor Štys, Jindřich Hašek

Lectures - Thursday, March 16

L1

REGULATION BY MOLECULAR INTERFERENCE: HOW 14-3-3 PROTEIN CONTROLS ACTIVITY OF FORKHEAD TRANSCRIPTION FACTOR FOXO4

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The 14-3-3 proteins, a family of dimeric regulatory proteins, are involved in many biologically important processes. The common feature of 14-3-3 proteins is their ability to bind to other proteins in a phosphorylation-dependent manner. Through these binding interactions, 14-3-3 proteins work as molecular scaffolds, modulating the biological functions of their partners. 14-3-3 proteins recognize short motifs containing a phosphorylated serine or threonine residue. FOXO transcription factors are promising candidates to serve as molecular links between longevity and tumor suppression. These factors are major substrates of the protein kinase B (Akt). In the presence of insulin and growth factors, FOXO proteins are relocalized from the nucleus to the cytoplasm and degraded via the ubiquitin-proteasome pathway. In the absence of growth factors, FOXO proteins translocate to the nucleus and upregulate a series of target genes, thereby promoting cell cycle arrest, stress resistance, or apoptosis [1,2].

Under conditions of low protein kinase B (PKB) activity (in the absence of growth factors), FOXO proteins are predominantly nuclear and the rate of import exceeds the rate of export. This shift in equilibrium probably occurs because the binding of FOXO to DNA anchors FOXO within the nucleus. Following the activation of PKB (PKB-regulated shuttling) by insulin-phosphatidylinositol-3-kinase (PI3K) signalling, PKB translocates to the nucleus. A 14-3-3 protein devoid of ligand enters the nucleus passively. PKB phosphorylates FOXO, probably on multiple sites, and PKB-mediated phosphorylation induces the binding of 14-3-3 protein to the FOXO proteins in the nucleus, which results in the release of the FOXO protein from the DNA. Following translocation to the cytosol, the bound 14-3-3 protein prevents re-entry into the nucleus by masking the NLS and inhibiting importin binding. This results in an equilibrium shift towards the cytosol.

Main goal of our research was to elucidate the molecular mechanism of 14-3-3 protein-dependent regulation of FOXO4 function. We have shown that phosphorylation of

FOXO4 by protein kinase B at Thr-28 and Ser-193 creates two 14-3-3 binding motifs. Analytical gel filtration and sedimentation equilibrium experiments indicate that doubly phosphorylated FOXO4 and 14-3-3zeta form a complex with 1:2 molar stoichiometry and a $K(D)$ of less than 30 nM. An active role for 14-3-3 in the disassembly of the FOXO4/DNA complex is demonstrated by the fact that, in the presence of 14-3-3, two phosphorylated 14-3-3 binding motifs are needed for the complete inhibition of FOXO4 binding to its target DNA [3]. We have also investigated whether the phosphorylation by protein kinase B, the 14-3-3 protein, and DNA binding affect the structure of FOXO4 nuclear localization sequence (NLS). We have used site-directed labeling of FOXO4 NLS with the extrinsic fluorophore 1,5-IAEDANS in conjunction with steady-state and time-resolved fluorescence spectroscopy to study conformational changes of FOXO4 NLS *in vitro*. Our data show that the 14-3-3 protein binding significantly changes the environment around AEDANS-labeled NLS and reduces its flexibility. On the other hand, the phosphorylation itself and the binding of double-stranded DNA have a small effect on the structure of this region [4].

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L2

OPTICAL SPECTROSCOPY, MASS SPECTROMETRY AND NMR ARE ESSENTIAL TOOLS IN THE PRODUCTION OF SOLUBLE RECEPTORS OF NATURAL KILLER CELLS

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Natural killer (NK) cells represent an important lymphocyte subset critical in the immune response against viruses, certain microorganisms, and tumors. Effector functions of these cells are dependent on the balance between signals transmitted from both activating and inhibitory surface receptors [1]. While the inhibitory receptors interacting with MHC class I as ligand have been well characterized, much less is known about the activating receptors and their ligands [2,3]. In our laboratory we have been producing *soluble recombinant forms* of the two major activating receptors of NK cells, *CD69* and *NKR-P1*. Our production protocol includes bacterial expression of the extracellular portion of the above receptors. Precipitation into the inclusion bodies is followed by *in vitro* refolding and purification by a combination of ion exchange, hydrophobic, and gel permeation chromatographies. The purified proteins appeared to be *homogeneous on SDS polyacrylamide gel electrophoresis* displaying a notable shift towards higher mobility under the nonreducing conditions. They are refolded in the form of *noncovalent dimers* as revealed by gel filtration and cross-linking experiments. The verification of the proper folding after *in vitro* procedure is critical for all the subsequent protein applications. For the *initial assessment* of the refolding efficiency, we use a combination of the *UV, FT-IR* and *Raman spectroscopy*. UV spectroscopy serves as a good first test of the stability of the produced protein (thermal denaturation curves measured in varying chemical environments). Vibrational spectroscopy, on the other hand, is able to estimate the content of secondary structure elements (such as α -helices, β -sheets etc.) as well as structural details of certain amino acids such as cysteines. For the *final verification* of the folding status, *high resolution methods* that look onto proteins in the high

magnetic fields are necessary. We use ion *Fourier transform-ion cyclotron resonance mass spectrometry* to look at the identity of the entire protein and the number of closed disulfide bonds. Measurement of the *1H – 15N HSQC spectra in a 600 MHz NMR spectrometer* using the uniformly ¹⁵N labeled protein provides the definitive evidence for the proper folding and long-term stability of the protein preparations. The above measurements revealed that our preparations of soluble *rat and human CD69 protein* is stable for weeks / months at temperatures between 4 °C and 30 °C, while the short term stability up to 80 °C is worth mentioning [4]. Also, our most recent measurements of *1H – 15N HSQC spectra of the rat NKR-P1A protein* points to the success of the currently employed refolding protocol. The use of NMR as well as protein crystallography and other methods for the ligand identification and verification experiments is under progress.

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L3

A SINGLE-POINT MUTATION OF M-PMV MATRIX PROTEIN CAUSES REORIENTATION OF PROTEIN DOMAINS AND CHANGES THE PHENOTYPE OF THE VIRUS

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Mason-Pfizer monkey virus (M-PMV) is a representative member of D-type retrovirus family. Characteristic feature of this family is the location of assembly of new virions which occurs in the pericentriolar region of an infected cell. It is the matrix protein (MA) – the N-terminal part of Gag polyprotein – that is responsible for directing the assembly of Gag polyproteins to the site of assembly [1]. MA protein interacts with Tctex-1, the light chain from a dynein molecular motor, and the Gag can then be transported in a complex with dynein [2]. If we introduce mutation R55F or R55W to the MA, the viral capsids are no longer assembled in the pericentriolar region, but at the inner side of the plasma membrane [3]. This modified behavior is typical for C-type retroviruses (e. g. HIV-1).

We present here 3D structures of the wild type MA and its R55F mutant solved by NMR spectroscopy. The com-

parison of both structures revealed a completely different orientation of N- and C-terminal domains, while the local structure within the domains remained conserved. The re-orientation causes that part of the linker (residues 42–52) between both domains becomes hidden, therefore the interaction with Tctex-1 is prevented. The results are supported by ¹⁵N relaxation measurements.

We thank the Grant Agency of the Czech Republic for the financial support (Grant 203/03/0490).

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L4

NMR PARAMETERS IN RNA MOLECULES AND THEIR CORRELATION WITH MOLECULAR STRUCTURE

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Structure of nucleic acids (NAs) is described by several structural parameters: six torsion angles () in the backbone, sugar conformation (C2'-endo or C3'-endo), and glycosidic torsion angle () which designate orientation of nitrogenous base relative to sugar moiety.

– O3' – P – O5' – C5' – C4' – C3' – O3' – P – O5' –

We studied correlation between NMR parameters and the local architecture of distinct patterns of RNA dinucleotides.

Model compounds of RNA were studied by quantum chemistry calculation methods with the aim to obtain NMR shifts and spin-spin coupling constants. We used different atomic bases Iglo II or Iglo III and water solvent was mod-

eled by the PCM method. Calculated NMR parameters, in particular the J-couplings, were correlated with the structure of RNA dinucleotides. In addition we monitored influence of nitrogenous bases on J-coupling in the backbone.

The three bond J-coupling constants can be used for the determination of torsion angles. Our calculations were compared with available data from the literature as well as with empirical Karplus curves.

The torsion angles can be naturally grouped according to their similar correlation with relevant NMR parameters: with with and with Torsions and can be determined by the coupling constants of the type ³J(C,P) and ³J(H,P) which are frequently measured. Torsion angles and can be correlated with the ³J(H,H), ³J(H,C) coupling constants. Torsions and can't be associated with any



three-bond coupling constants that would unequivocally describe their values but we were able to identify their weak correlation with the two bond coupling constants ${}^2J(C,P)$.

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PL1

BIONMR: CONTEMPORARY TRENDS, CHALLENGES, AND FUTURE PROSPECTS

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Nuclear Magnetic Resonance (NMR) spectroscopy represents an invaluable tool to investigate the structure and dynamics of biomolecules. Biological NMR is experiencing significant development with new hardware and software tools as well as new methodological approaches appearing on the scientific arena. During the past decade, the most influential developments can be listed as follows: employment of residual anisotropic interactions in partially oriented biomolecules, improvement of isotope labeling strategies for proteins, RNA, and DNA molecules, identification of cross-hydrogen bond spin-spin interactions, sensitivity and resolution enhancement of NMR signals by

cross-relaxation compensation, fast data acquisition methods, improvement of dynamics characterization by analyzing the relaxation parameters, introduction of 900 MHz NMR systems, employment of cryogenically cooled probes with greatly enhanced sensitivity, and advancement of protein structure determination using solid state NMR on polycrystalline samples. Along with those achievements, NMR is shifting its focus from "simple" high-throughput structure determination to more functionally oriented applications. The lecture will critically review the recent progress of high-field NMR and will outline the possible developments expected within next few years.

Lectures - Friday, March 17, morning

PL2

METHODS OF OPTICAL SPECTROSCOPY FOR STRUCTURAL BIOLOGY

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Methods of optical spectroscopy represent a useful tool to study structure and dynamics of biomolecules in solution. Although these techniques cannot yield 3D structures of studied objects with atomic resolution they can provide answers to specific questions. In this lecture, basic principles

of various methods based on interaction of molecules with ultraviolet, visible and infrared radiation will be briefly summarized and then several representative applications will be presented to illustrate their usefulness, strength and in some respects even uniqueness.

L5

DNA REPLICATION FIDELITY: THEORY AND EXPERIMENT

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Understanding the origin of the fidelity of DNA polymerases is a problem of enormous importance. Despite great experimental progress we still do not have a detailed molecular picture of the factors that control the fidelity.

Here we describe the application of computer simulation approaches [1] to gain a deeper insight into fidelity mechanisms and to complement the experimental advances in the field. We focus on the calculations of structure-function

(fidelity) correlation and on the improvements of the obtained results by a constant feedback from kinetic, binding, and structural experiments.

Support from NIH (Program Project grant #1U19CA105010) is gratefully acknowledged.

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L6

MOLECULAR DYNAMICS STUDY OF MAJOR URINARY PROTEIN-PHEROMONE INTERACTIONS: A STRUCTURAL MODEL FOR LIGAND-INDUCED FLEXIBILITY INCREASE

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Recently, two independent ^{15}N NMR relaxation studies [1,2] indicated that in contrast to the decreased flexibility expected for induced-fit interactions, the backbone flexibility of major urinary protein isoform I (MUP-I) slightly increased upon complex formation with its natural pheromone 2-sec-butyl-4,5-dihydrothiazol. We have analyzed motions in terms of frequency-dependent and conformation-dependent order parameters S^2 by molecular dynamics simulations of free- and complexed protein. In general, calculated order parameters S^2 agree with the NMR relaxation data. Structural analysis supplied evidence that mobility in different regions can be attributed to

small conformational changes of free- and complexed protein, which results from hydrogen bonding network modifications [3].

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L7

MD AND AB INITIO MODELING OF ELECTRONIC SPECTRA OF N-METHYLACETAMIDE AND PEPTIDES IN WATER SOLUTIONS

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Many methods used in analytical chemistry are based on interactions of molecules with ultra-violet, visible (UV-vis) or infrared (IR) light. Spectral shapes and band positions depend not only on the structure, but also on solvent environment. We deal with modeling of absorption and circular dichroism (CD) spectra in UV-vis region and with the influence of polar solvents (water). *N*-methylacetamide (NMA) is the simplest molecule containing the amide chromophore R-CO-NH-R' (where R and R' are other groups than H) and thus it is convenient for modeling of general spectral characteristics of peptides and proteins [1]. The influence of the solvent was simulated by combining of continuum dielectric model (COSMO) with inclusion of explicit water molecules. Results of these calculations revealed three main mechanisms by which the solvent modifies the spectra: 1) *via* change of geometrical parameters of the amide group, 2) by the electrostatic field (in the case of polar solvent) and 3) by the hydrogen bonding and direct participation of the water orbitals.

Cluster geometries were obtained from MD simulations (TINKER software, AMBER forcefield). Cluster absorption spectra were computed *ab initio* in vacuum and with the COSMO model for multiple geometries. Average spectra were in a very good agreement with experimental spectrum of aqueous NMA solution. Influence of non-H bonded water molecules on the band shapes and frequencies was not significant.

Average transition dipole moments of the NMA molecule obtained *ab initio* were used for the predicting of the CD spectra (in the UV region) of main peptide conformations: -helix, -sheet and coil conformation (polyproline II-like). Rotatory strengths were obtained by a semiempirical Transition Dipole Coupling (TDC) model. Computed CD spectra of -helix and coil were in a good agreement with available experiment, for -sheet the agreement was not so good.

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L8

MOLECULAR MODELLING STUDIES WITH BOWMAN BIRK INHIBITORS

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Protein homology molecular modelling combined with energy minimisations is used as a useful tool for obtaining insight into small structural perturbations as a result of amino acid replacements in the Bowman Birk inhibitor of serine proteases. This model protein built up from a relatively short amino acid chain of 71 residues contains a binary arrangement of a trypsin- and chymotrypsin-inhibitory subdomain and a rigid structural framework of seven disulfide bonds. We have performed molecular modelling studies with several homologues of BBI as an independent test for the validity of this approach followed by calculat-

ing models of different site directed mutants of the inhibitor. The models displayed only minor structural deviations with regards to the parent protein. These deviations were none the less extremely useful with regards to the irregularities in the variants monitored by comparative titration- and activity determination experiments in the two subdomains. These experimental and theoretical data will be used in order to derive a structural model of a coupled rather than independent folding of the two subdomains in the Bowman Birk inhibitors.

L9

THE USE OF GPU BASED CALCULATIONS IN STRUCTURAL BIOLOGY

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There still exist a lot of very computational extensive task in both structural biology and crystallography. Typical examples of problem requesting extreme computational power are QM calculation, molecular dynamic simulations or structure solution from powder data.

The latest development in computer graphic HW gives us an alternative to the classical computational model based on single CPU or clusters of PC.

The Fig.1 demonstrates clearly the nowadays differences between the computational power of graphic cards and single core or dual core standard CPU. The trend is clear – the already existing graphic cards have up to 20 times higher brute force computational power in comparison to existing CPU. The main reason of such a speed dif-

ference is computation distribution between multiple GPU processors inside the graphic card and the use of very fast memory elements as well as support for HW implemented vector instructions.

Unfortunately utilization of GPU for normal calculations is non trivial. The algorithm must operate with the multi processor structure of the GPU as well as with several data operation limitations inside the graphic card. In the area of structural biology the GPU based calculations were experimentally used for solving of following problems: Molecular dynamic simulation [2], HMM Viterby probability search in structure databases [3] and NMR-distance smoothing for protein structure determination [4].

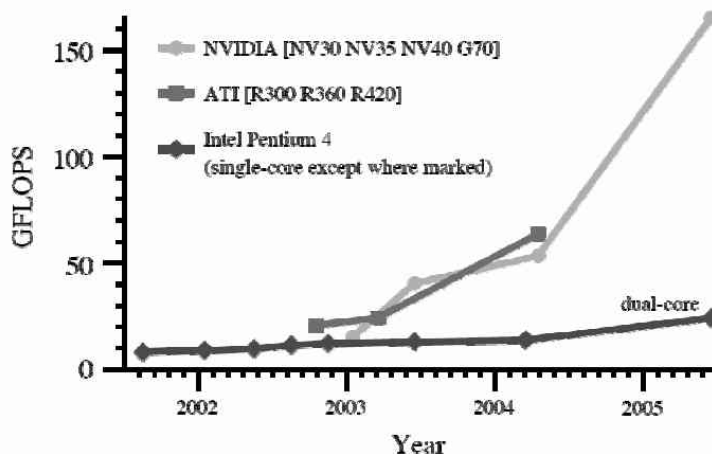


Fig. 1.

In our department we had made only some proof of the concept calculation. We had repeated the NMR-distance smoothing algorithm [3] benchmarks and we had made very simple benchmarks based on several trivial mathematical calculations.

Generally the GPU based calculation is a promising tool for drastic speed up of important computations. To fully utilize it for the structural biology needs it is necessary to modify existing algorithm to versions able to use parallel processing. There still exist several principal limits related to the nature of the graphic HW making GPU non suitable for several key operations (as e.g. large linear equations system solving). The reason is low computation precision in GPU and different data caching model in comparison to CPU. It is expected that such problems will be handled in new graphic cards optimized not only for

graphic operations, but also for potential use as computational units.

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L10

BACTERIAL PROGRAMMED CELL DEATH – GENETICAL, BIOCHEMICAL, AND STRUCTURAL INSIGHT

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Programmed cell death in bacteria, as it analogue in eukaryotes, is defined as active process, which during the development or due to environmental signals leads to death of a particular cell.

The examples of programmed cell lysis during the developmental processes are mother cell lysis of sporulating *Bacilli*, fruiting body formation of *Myxococcus* bacteria, and colony rearrangement of *Streptomyces* species. On the other hand, genes responsible for programmed cell death as a consequence of effect of various harmful agents from an environment have been found in wide range of prokaryotes. It has been shown that a presence of these agents starts a self-destruction program of seriously affected cells, rather than killing the cells by the agents themselves. The act of suicide is in, these cases, usually performed by a stable toxin protein, effect of which is otherwise neutralized by its unstable antidote. Transcription of both genes coding for toxin and its antitoxin, which can be protein or untranslated antisense RNA, is frequently regulated from one common promoter. Such systems have been first identified stabilizing plasmid DNAs bearing corresponding genes. However, presently there are known several toxin-antitoxin families, cassettes of which are present on chromosomes of variety of bacteria.

There are only few identified cellular targets of toxin, but in all instances the toxicity directs either the cytoplasmic membrane integrity, or the replication and translation machineries, which are all factors crucial for cell viability. In few cases the mechanisms of toxicity is known even at the molecular level, shading more light to our understanding of processes affected by the toxin.

Out of dozen main groups of toxin-antitoxin systems, molecular structure of five toxin and three antitoxin pro-

teins are already known. In some cases, these structures bring many interesting information about the way how a toxin affects function of its cellular target, or how its activity is regulated through the binding of the antidote. However, in majority of these systems, structures of toxin-antitoxin and toxin-target complexes have to be solved. This is still sometimes complicated by the fact that the cellular targets of several toxins are not identified yet. Instability of antitoxin proteins are frequently due to their degradation by specific proteases. Thus it makes them an interesting subjects for biochemical and structural studies.

In our work we focused on genetical, biochemical and structural studies of components of SpoIIS toxin-antitoxin system from *Bacillus subtilis*. SpoIISA toxic protein comprises two domains – N-terminal membrane spanning and C-terminal cytosolic parts. Our results indicate that neither of these two domains alone is sufficient for toxic effect. However, the oligomerization driven by cytosolic parts is needed for the toxicity of SpoIISA. This oligomerization seems to be blocked by tight binding of basic SpoIISB antitoxin to negatively charged C-terminal part of SpoIISA. To further appreciate the mechanisms of this regulation we attempted to crystallize both proteins and their complex.

The work in author's laboratory is supported by grant 2/1004/21 from the Slovak Academy of Sciences and by grant APVT-51-027804 from Ministry of Education of Slovak Republic.

**Lectures - Friday, March 17, afternoon****PL3****MOLECULAR SYSTEMS BIOLOGY: MOLECULAR SIMULATIONS OF PROTEINS AND THEIR COMPLEXES****R. Ettrich**

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Simulation on a molecular level has evolved to become the tool of choice to model the physical properties of complex biological systems. Molecular dynamics simulations as such have become the “workhorse” of structural biologists. Ten years ago state of the art simulations could barely access the nanosecond time scale and the increase in cpu power has pushed this limit two orders of magnitude. Interestingly, the increase in power of simulations is only partly due to the large increase in computing power during the past 50 years. Development of novel computational techniques extend molecular simulations towards longer time and length scales in molecular systems biology. Three-dimensional structures to build up a stable complex biologi-

cal system that might be studied by means of molecular dynamics are gained either by experimental techniques of X-ray diffraction and protein NMR or by homology and energetic modeling in combination with low-resolution spectroscopic techniques as are circular dichroism, Raman spectroscopy or FTIR.

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L11**A CORRELATION-BASED APPROACH FOR PREDICTING PROTEIN DOMAINS FROM SEQUENCE****M. Lexa**

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Protein structure prediction from sequence is a very important problem that has not been solved satisfactorily yet. Prediction is possible for some sequences by the means of homology modelling, threading, fragment assembly or ab-initio modelling. An important step of any prediction method is preprocessing of the analyzed sequence to recognize the presence of possible domains. If domains can be identified, the problem can be split into a set of simpler problems, each dealing with one domain separately. Domains often contain typical regions of sequence similarity to other proteins containing the same domain, therefore similarity searches represent a natural approach to domain identification. This has been used, for example, in the construction of the ProDom database of protein domains [1]. Unfortunately, many sequences lack sequence similarity to existing proteins, and domains can not be identified this way. An alternative method based on a statistic recognizing interdomain regions, rather than the domains themselves, predicts domain boundaries from the amino acid composition of the analyzed sequence regardless of the presence or absence of sequence similarity [2]. Another method predicts the boundaries of domains by considering the hydrophobicity of aminoacids and their likelihood to occur inside or outside a typical globular domain [3].

In this contribution, I present a new approach to domain prediction. While based on sequence similarity as some previous methods, it only relies on very short segments of sequence similarity that might be considered noise by many other methods. The novelty and potential power of this method lies in the subsequent analysis of the identified short similarities. These are considered in pairs occurring along the analysed sequence. Pairs that are found correlated in a large protein database are assumed to have some common function (structural or other) in the protein. As such, they are much more likely to exist within a single domain, since domains are often considered to be units of elementary functions. Regions of the analyzed sequence spanned by many such correlated pairs are then evaluated as domain candidates. Regions with a minimal number of spanning correlations are predicted to be domain boundaries. Compared to previously reported version of this approach [4], a clustering step has been added to the analysis, to enable detection of non-contiguous domains.

The new method is being tested against a database of proteins with known domain composition. Other methods will be compared to our methods and the results presented.



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L12

CRYSTAL STRUCTURE OF PHOTOSYSTEM II FROM *Thermosynechococcus elongatus* REFINED AND STUDIED BY MOLECULAR DYNAMICS

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Crystal or NMR structures are essential and fundamental in performing almost all molecular modelling techniques. Three dimensions resolution of such structures is certainly one of the most crucial criteria of quality and credibility. Researchers made great effort to prepare crystals of photosystem II (PS II) from algae and higher plants in the last decades. However, till now there are only two experimental crystal structures resolved at adequate resolution. Both are from the same common organism *Thermosynechococcus elongatus*. First was obtained at 3.5 Å (PDB code: 1S5L) [1] and second at 3.2 Å (PDB code: 1W5C) [2] overall resolution. By performing series of molecular dynamics (MD) simulations at appropriate time scales also coupled partially with quantum-chemical calculations, it is possible to increase the model accuracy mainly in the regions, where the probability of spatial orientation of amino acid side chain lacks appropriate electron density or other sources of experimental data. We present here more natural-like, geometrically-optimised structures of extended reaction centre (RC) of PS II.

First, we constructed truncated models of PS II considering protein subunits and pigment molecules from and around the RC, keeping also functionally and structurally important spatial subunits. These structures were then used as starting structural models for MD simulation runs. Force field (FF) [3] parameters (charge distribution and force constants) and topology for PS II RC pigment molecules [4] were developed for the Yamber2 FF [5, 6] by performing quantum chemical calculations and by modifying and extending Ceccarelli [7] studies concerning bacterial type *a* pigments. Geometry optimisation of PS II RC pigment molecules and development of new FF parameters was performed at RHF/6-31G* level of quantum chemical theory using Gaussian 98 [8]. Calculation of RESP atomic charges following AMBER FF developing scheme [9] was done using Gaussian 98, R.E.D, antechamber, and resp subroutines. Complete FF parameters and topology for the PS II RC pigment molecules were developed and introduced to

YASARA [5]. Several MD simulation runs, taking into account different pigment oxidation states and various solvent properties, were performed in order to check the quality of new FF parameters for the PS II RC pigments. Subsequently, by further optimising overall geometry of new structural models using MD simulation runs with small time step of 1.0 fs, we obtained final models of PS II RC complex. Detailed analysis of new FF parameters showed realistic dynamic behaviour of pigment molecules and typical electrostatic interactions with the surrounding protein environment.

Recently, changes in excitonic interactions of PS II RC pigments upon light-induced oxidation of primary donor (P680) or reduction of primary acceptor pheophytin *a* (Phe *a*), were analysed using absorption and circular dichroism (CD) spectra [10, 11]. In contrast to the oxidation of primary donor, the light-induced change in the CD spectrum upon primary acceptor reduction was temperature-dependent. This suggests a hypothesis that at a room temperature the reduced Phe *a* induces conformational changes of the RC protein environment, which affects the excitonic interaction of the RC chlorophylls (Chls). Having optimised structural models of PS II RC we were able to elucidate and describe some of the details of these processes.

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L13

FROM NONPEPTIDE TOWARD NONCARBON PROTEASE INHIBITORS: METALLACARBORANES AS SPECIFIC AND POTENT INHIBITORS OF HIV PROTEASE

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Jana Pokorná¹, Jaromír Plešek⁵, Bohumír Grüner⁵, Lucie Dolečková-Marešová¹,
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HIV protease (PR) represents a prime target for rational drug design, and protease inhibitors (PI) are powerful antiviral drugs. Most of the current PIs are pseudopeptide compounds with limited bioavailability and stability, and their use is compromised by high costs, side effects, and development of resistant strains. In our search for novel PI structures, we have identified a group of inorganic compounds, icosahedral metallacarboranes, as candidates for a novel class of nonpeptidic PIs. Here, we report the potent, specific, and selective competitive inhibition of HIV PR by substituted metallacarboranes. The most active compound, sodium hydrogen butylimino bis-8,8-[5-(3-oxa-pentoxo)-3-cobalt bis(1,2-dicarbollide)]di-ate, exhibited a K_i value of 2.2 nM and a submicromolar EC_{50} in antiviral tests, showed no toxicity in tissue culture, weakly inhibited human cathepsin D and pepsin, and was inactive against trypsin, papain, and amylase. The structure of the parent cobalt bis(1,2-dicarbollide) in complex with HIV PR was

determined at 2.15 Å resolution by protein crystallography and represents the first carborane-protein complex structure determined. It shows the following mode of PR inhibition: two molecules of the parent compound bind to the hydrophobic pockets in the flap-proximal region of the S3 and S3' subsites of PR. We suggest, therefore, that these compounds block flap closure in addition to filling the corresponding binding pockets as conventional PIs. This type of binding and inhibition, chemical and biological stability, low toxicity, and the possibility to introduce various modifications make boron clusters attractive pharmacophores for potent and specific enzyme inhibition.

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic (MSM60076658 08, GACR206/02/D177) and by the Academy of Sciences of the Czech Republic (Institutional research concept AVOZ 60870520 and AV0Z50510513).

L14

STRUCTURAL STUDIES OF GALECTIN-4 N-TERMINAL DOMAIN IN COMPLEX WITH LACTOSE

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Galectins are an evolutionarily conserved family of 15 different lectins found in various combinations in virtually every type of animal cell. One of the primary galectins expressed in intestinal epithelium is galectin-4, a tandem-repeat galectin with two carbohydrate-recognition domains in a single polypeptide chain having affinity for β -galactosides as the lactose [1].

We reported here the X-ray structure of mouse galectin-4 N-terminal domain (CRD1) in complex with lactose (Figure 1) at resolution 2.1 Å. Comparison with other galectins, mode of ligand binding and fluorescence of interacting Tryptophan will be discussed.

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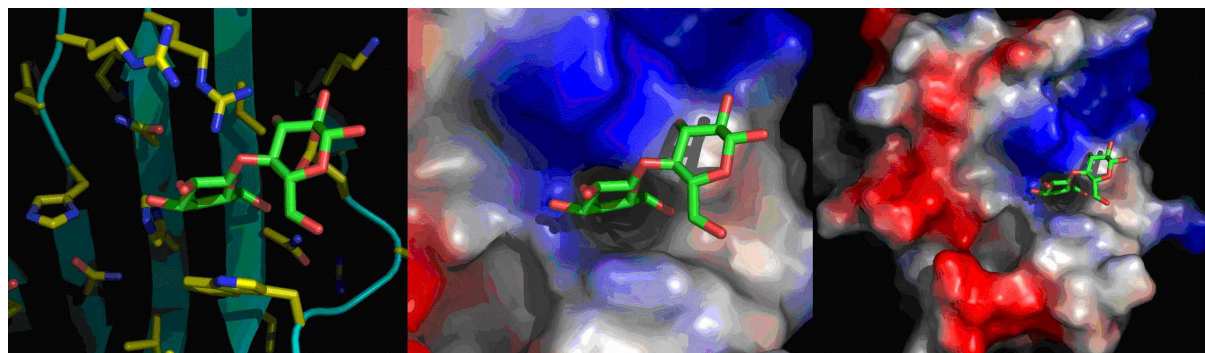


Fig. 1. a) Detail view of interacting lactose (carbons coloured green) in binding site. b) The same as a), solvent accessible surface of galectin is coloured by electrostatic potential (negative red, positive blue).

L15

DNA CONFORMATIONAL CLASSES

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7753 dinucleotides from 447 high resolution DNA structures were analyzed in the torsional space of 14 conformational variables. The conformational types were determined by the following stepwise classification procedure. The first step involves the choice of the 3D maps of torsion angles (9 different combinations of torsion angles were used), and the identification of data points aggregates (peaks) based on their density by the means of Fourier averaging. In each of the nine analyzed maps, ~20 peaks were identified. Each peak was approximated by a sphere,

and individual data points were assigned, based on their distances from peak centers, to the peaks. All data points were labeled by names of the neighbouring paks in all nine maps, and they were, in the second step, clustered by a technique called lexicographical clustering. Lexicographical clustering creates a typical imprint for each data point; an identical (or close to identical) imprint of a group of data points then defines a cluster. Because each imprint represents a conformation near peak positions, each cluster then represents a dinucleotide conformational family.



PL4

STRUCTURE AND DYNAMICS OF RNA AND DNA. ADVANCED COMPUTATIONAL STUDIES

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Modern computational approaches represent a useful tool to study selected aspects of structure and dynamics of nucleic acids. I will present a short survey of the latest developments in the field, with a special emphasize given to

molecular dynamics studies of RNA and DNA. Basic principles, main advantages and substantial limitations/failures will be explained.

Lectures - Saturday, March 18, morning

PL5

STRUCTURE ELUCIDATION BY DIFFRACTION METHODS – CONTRIBUTION TO STRUCTURAL BIOLOGY

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The first documented X-ray scattering on ordered samples of biological origin was observed by J. D. Bernal in Cambridge, U.K. in 1934. Further experiments by Dorothy Crowfoot-Hodgkin and M. F. Perutz in Oxford on crystals of insulin and haemoglobin in 1937 and in the following years lead to development of a method which revolutionized our views of biochemical processes and molecular structure of living systems. The number of structures determined by diffraction methods from 1960 to these days (the first one of myoglobin by Kendrew) amounts to tens of thousands, many of them remaining unpublished or subject to proprietary rights of private companies. The length of the process of solving and finalizing a structure dramatically decreased from several years to several weeks or months depending on the project difficulty.

While from 1950s till 1980s methods for structure solving such as molecular replacement and isomorphous replacement were developed, late 1980s and 1990s saw a fast development of computational technology and its application in all steps of macromolecular structure determination. In the recent ten years this field profited from huge development effort in crystallographic software as well as methodological advances in protein and nucleic acid crystallization and in data collection and analysis. Macromolecular crystallography today would be unthinkable without cryo-cooling of samples, synchrotron sources of high intensity X-ray radiation and high speed internet services and databases.

Crystallographically determined structures of biological molecules bring invaluable information ranging from details of interactions between ligands and enzymes, assemblies of protein-protein or nucleic acid – protein types to highly complex structures of viruses. Quality of acquired structural information does not depend on the size of the system once regularly organized.

The most recent advances (5 years) target the main bottlenecks of these methods and indicate future development. High throughput methods for protein production, purification and crystallization, robotized home source and synchrotron beam-lines and software equipment for automatic information-with-sample transfer and for automatic data collection were produced mainly within structural genomics projects for which the main starting impulse were the results of successful genomics projects. Systematic studies on radiation damage of biological samples induced by intensive radiation sources contributed substantially to better experiment planning and techniques. Free electron laser and single particle imaging are becoming centres of interest mainly for diffraction studies of larger systems (organelles or cells) without the need of an ordered crystal-line system.

Introduction of modern methods is supported by the Ministry of Education of the Czech Republic (1K05008).

L16

DISCOVERY OF STEREOSELECTIVE HALOALKANE DEHALOGENASE: NEW TOOL FOR ASYMMETRIC SYNTHESIS

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The spectrum of classical synthetic methods is significantly widened and enriched by expansion of biochemical methods. Enzyme-catalyzed reactions have become popular alternatives to classical organic chemistry for its high selectivity and activity under mild reaction conditions and several industrial processes using enzymes as a catalyst are already in use. Haloalkane dehalogenases (EC 3.8.1.5) are one class of such enzymes holding high potential for application in asymmetric biocatalysis. The main products of haloalkane dehalogenase reactions (alcohols) are valuable building blocks in organic synthesis and these enzymes do not need any cofactor or metal ion for their activity [1]. However, there has been no report that a member of this specific enzyme family shows sufficient stereoselectivity for production of optically active compounds. In 2001, Pieters and co-workers [2] have investigated enantioselectivity of haloalkane dehalogenase DhIA from *Xanthobacter autotrophicus* GJ10 and DhaA from *Rhodococcus rhodochrous* NCIMB 13064. The magnitude of the chiral recognition was low; a maximum E-value of 9 was reached after a structural optimization of the substrate. In 2004,

twenty years after discovery of the first haloalkane dehalogenase, development of enantioselective dehalogenases for use in industrial biocatalysis was defined as one of the major challenges of the field [3].

Hydrolytic dehalogenation of a series of racemic substrates by using three different enzymes has been performed in this study. The enzymes included in testing were previously studied DhaA, and two additional enzymes LinB from *Sphingobium japonicum* UT26 [4] and DbjA from *Bradyrhizobium japonicum* USDA110 [5]. The magnitude of the chiral recognition was low for most of the substrates, however excellent enantioselectivity of all three enzymes was observed in reaction with brominated esters of propionic and butyric acids. This observation demonstrated for the first time that a member of haloalkane dehalogenase family possesses sufficient enantioselectivity for synthesis of optically pure compounds. Furthermore, haloalkane dehalogenase DbjA showed high enantioselectivity also with α -substituted bromoalkanes. This enantioselectivity is interesting and surprising in light of a simple structure of this compound giving a very few

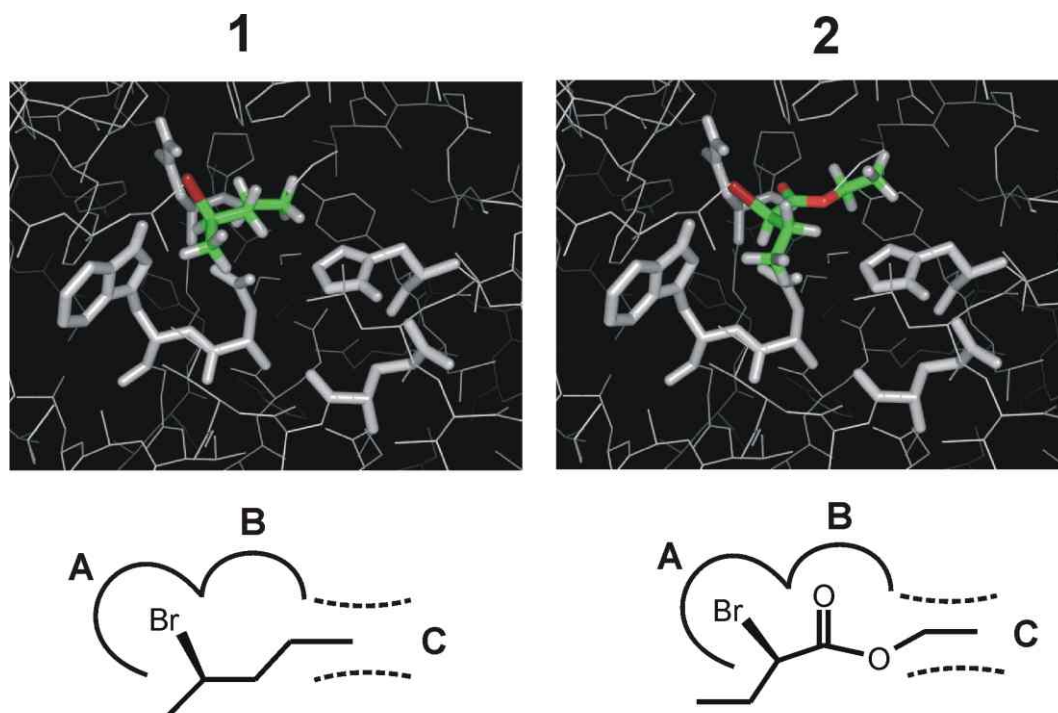


Figure 1 Active-site model of the haloalkane dehalogenase DbjA. Binding of two types of substrate molecules: α -substituted alkanes (1) and α -substituted esters (2). A, halide-binding site; B, oxygen-interacting site; C, alkyl-chain binding site. Catalytic pentad (in white) and substrate molecules docked in Michaelis complex (in color) are shown in stick.



possibilities for differential binding and catalysis of its enantiomers. On the other hand, -substituted esters have an extra group that can form additional hydrogen bond with the active site residues and make their binding more oriented. The resolution of esters could be achieved by three-point-attachment mechanism, which would not however apply to -substituted alkanes (Figure 1). A hypothesis of two separate mechanisms of haloalkane dehalogenase enantioselectivity towards -substituted bromoalkanes and -substituted esters has been proposed based on these observations. The extended substrate mapping together with molecular modeling brought the first view on mechanism of haloalkane dehalogenase enantioselectivity. Understanding of these mechanisms is essential for engineering of new enantioselective biocatalysts.

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L17

STRUCTURE-ENANTIOSELECTIVITY RELATIONSHIPS OF HALOALKANE DEHALOGENASES

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Haloalkane dehalogenases (EC 3.8.1.5) are enzymes able to remove a halide from halogenated aliphatic compounds by the hydrolytic replacement. Products of this reaction are corresponding alcohols. Recently we have demonstrated for the first time that certain proteins from this enzymatic family possess sufficient enantioselectivity for synthesis of optically active compounds. There is a high potential for an application of haloalkane dehalogenases in asymmetric biocatalysis, since optically active haloalkanes and alcohols are valuable building blocks in organic synthesis of intermediates for pharmaceuticals, agrochemicals, food additives, and odorants.

Hydrolytic dehalogenation of wide range of racemic substrates catalysed by haloalkane dehalogenases DhaA from *Rhodococcus rhodochrous* NCIMB 13064, LinB from *Sphingobium japonicum* (formerly *Sphingomonas paucimobilis*) UT26 and DbjA from *Bradyrhizobium japonicum* USDA 110 have been tested in this study. Three different haloalkane dehalogenases possess excellent

enantioselectivity with brominated esters (E-value > 200). Additionally, the haloalkane dehalogenase DbjA revealed high enantioselectivity towards -substituted bromoalkanes. This enantioselectivity is interesting due to the simple structure of these chemical compounds giving a very few possibilities for different binding and catalysis of (*R*) and (*S*) enantiomers. Sequence comparisons and structural analysis revealed the presence of insertion fragment unique to DbjA that is located on protein surface. Mutagenesis followed by kinetic characterization of deletion mutant confirmed that this fragment is involved in enantioselectivity of DbjA with -substituted bromoalkanes, but not with esters. This evidence suggests that DbjA enzyme is acting by two different enantioselectivity mechanisms. This study demonstrates that enantioselectivity of enzymes can be modulated by the engineering of surface loop which may have important implications for construction of new enantioselective biocatalysts.

L18

PHYSICAL VARIABLES AS A CONTROL OF ENZYME STEREOSELECTIVITY**R. Chaloupková, Z. Prokop, M. Straková, T. Mozga and J. Damborský***Loschmidt Laboratories, Faculty of Science, Masaryk University, 625 00 Brno*

The applicability of enzymes in an industrial processes for the production of fine chemicals requires that a number of criteria are fulfilled. Enantioselectivity is the most important when application in asymmetric biocatalysis is being considered. Many enzymes do not provide products of high stereomeric purity under physiological conditions. The manipulation of the physical environment is attractive way to provide an additional level of control of enzyme stereochemistry. In this work, the effect of temperature and pH on enzyme structure stability, activity and enantioselectivity was investigated. Haloalkane dehalogenase DbjA from *Bradyrhizobium japonicum* USDA110 [1] was selected as a model enzyme. The DbjA dehalogenase exhibits novel substrate specificity [1] and more importantly it is the first reported enantioselective haloalkane dehalogenase. High enantioselectivity of this enzyme was observed in reaction with selected α -substituted bromoalkanes and brominated esters [2].

Activity, enantioselectivity and conformational behavior of DbjA have been evaluated at different temperatures and pH. Conformational changes have been assessed by circular dichroism spectroscopy. Determined melting temperature T_m was 47.3 ± 0.2 °C. The DbjA enzyme is highly tolerant to pH changes. The secondary structure of DbjA was not significantly affected by pH in the range 5.3-10.3. At highly acidic conditions (pH < 5.3), the enzyme aggregates, whereas at extreme alkaline conditions (pH > 10.3) exist in predominantly disordered conformation. The temperature and pH optima of DbjA enzyme were determined with 1-iodohexane by activity measurement. Maximal activity was detected at 50 °C and at pH 9.7. The highest E

value (174) of DbjA enzyme towards 2-bromopentane was determined at 20 °C. The effect of pH on enantio-discrimination of 2-bromopentane by DbjA was not observed, while the temperature significantly altered enzyme enantioselectivity. Lowering of temperature from 50 to 20 °C results in thirteen times higher DbjA enantioselectivity towards 2-bromopentane. Racemic temperature as well as differential activation parameters enthalpy and entropy have been calculated based on relations between enantioselectivity and temperature. Racemic temperature was evaluated to be 85.6 °C, ΔH_{R-S} to be -69.5 kJ/mol and ΔS_{R-S} to be 0.2 kJ/mol. The enzyme preferentially catalyzed enthalpically favored (*R*)-enantiomer.

These observations demonstrate that haloalkane dehalogenase DbjA possess high activity and structural stability through wide range of pH conditions. Furthermore, the enantioselectivity of DbjA enzyme with selected substrate could be improved by decreasing experimental temperature. We conclude that physical variables represent an important and easiest approach for modulation of enzyme catalytic properties which can be explored in biotechnologies.

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L19

ENGINEERING OF ENANTIOSELECTIVE HALOALKANE DEHALOGENASE BY CUMULATIVE MUTAGENESIS**T. Chrobáková¹, Z. Prokop¹, Y. Sato², M. Monincová¹, A. Jesenská¹, L. Grodecká¹, T. Senda³, Y. Nagata² and J. Damborský¹**¹*Loschmidt Lab., Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Rep.*²*Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai, 980-8577, Japan*³*Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, 2-41-6 Aomi, Koto-ku, Tokyo 135-0064, Japan*
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Enantioselective enzymes are useful as catalyst for biosynthesis of various organic compounds. Members of the haloalkane dehalogenase family (EC 3.8.1.5), which belong to superfamily of α -hydroxylases, produce alcohols during the dehalogenating reaction. If dehalogenases are enantioselective, they could catalyze synthesis of several

alcohols important for pharmaceutical, food and agricultural industry. However, only DbjA from *Bradyrhizobium japonicum* USDA110 [1] is showing significant enantioselectivity towards several types of substrates. The objective of this project is to extend this interesting property to other family members. The most structurally related



haloalkane dehalogenase DhaA from *Rhodococcus rhodochromus* NCIMB 13064 was selected for mutagenesis.

Amino acids in the active site of DbjA, possibly determining enantioselectivity of this enzyme, were included in experimental design of reconstruction of enantioselective haloalkane dehalogenase DhaA. Residues were chosen based on sequence alignment of DbjA with DhaA. In addition to nine point substitutions (H105Q, W141F, P142A, F144A, G171R, A172V, K175G, C176G and V245A), the main difference is in the presence of the insertion loop (₁₃₉HHTEVAEEQDH₁₄₉) between the main and the cap domain of DbjA.

Seven rounds of mutagenesis were designed. At first, the mutant gene containing the loop-coding sequence was prepared by inverse polymerase chain reaction. Other mutations were cumulatively added using QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene, USA) according to manufacturer's instructions. Genes were amplified using specific complementary primers introducing substitutions into sequences. The genes carrying mutations were cloned into the pET21b expression vector (Novagen, USA) and expressed in *Escherichia coli* BL21 (DE3). Proteins were purified using HiTrapTM Chelating column with metal

affinity resin (Amersham Biosciences, Germany). Their molecular weight and purity were checked by SDS-polyacrylamide electrophoresis. Folding of proteins was verified by circular dichroism (CD) spectroscopy. The dehalogenating activity of enzymes towards 1,3-dibromopropane and stereospecificity with the racemic mixture of the set of α -substituted aliphatic bromoalkanes and α -halogenated ester were tested by gas chromatography.

Six mutant genes of haloalkane dehalogenase DhaA were successfully constructed. All proteins were produced in a soluble form. The yields of protein variants except the first mutant were high. Four out of six mutants were active towards 1,3-dibromopropane. Activity data were consistent with the changes that were observed in CD spectra. Last two mutants with the most similar CD spectra to DhaA showed activity comparable to the wild type enzyme. Testing of mutants for enantioselectivity is in progress. The results of these experiments will be presented during the lecture.

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MODELLING OF PRODUCT RELEASE AND IDENTIFICATION OF EXPORT ROUTES IN HALOALKANE DEHALOGENASE DHA A

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Halogenated aliphatic hydrocarbons are widespread recalcitrant compounds due to massive natural and industrial production. They are environmentally dangerous because of their toxic, genotoxic, teratogenic and irritating effects. Microbial enzymes haloalkane dehalogenases have been studied for decades for their possible use in bioremediation owing to their capability to catalyse hydrolytic dehalogenation of the xenobiotics to harmless products. Practical use of these enzymes requires increase of activity, specificity and thermostability through modification in their structures.

Three haloalkane dehalogenases with experimentally solved three-dimensional structure differ in rate-limiting step of the dehalogenation reaction. The slowest step for Rhodococcal haloalkane dehalogenase DhaA is release of an alcohol from the active site cavity to bulk solvent. The release may occur through two tunnels called the main tunnel and the slot. Rational computer-assisted re-design of the tunnels could lead to DhaA with higher catalytic activity. Classical molecular dynamics is, however, not appropriate tool for modelling of egress of the product of the reaction because this process is rare event and cannot be observed during nanosecond simulations. We applied Ran-

dom Accelerated Molecular Dynamics (RAMD) [1] to speed up the egress by applying a force on the product molecule. Using RAMD, we are capable to model export pathways in the time scale from tens to hundreds picoseconds.

RAMD simulations were performed with DhaA in complex with two products of dehalogenation of 1,2,3-trichloropropane, i.e. chloride, (R)- and (S)-2,3-dichloro-1-propanol (R- and S-DCL). Complexes were prepared by molecular docking and equilibrated using classical molecular dynamics. Chloride anion solvated by water molecules left the active site through main tunnel in the presence of S-DCL after 1.7 ns of equilibration phase of molecular dynamics. Its release has never been observed in simulations without alcohol and suggested that chloride leaves the active site with the assistance of water molecules and before DCL. RAMD applied on DCL showed two pathways for DCL, i.e. the main tunnel and the slot, the tunnel being preferred export route. Based on these observations we designed directed evolution experiments attempting to improve catalytic performance of DhaA by engineering of its export routes.

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