



## Posters

P1

### EXPRESSION AND PURIFICATION OF APO-MICRO-MYOGLOBIN AND ITS RECONSTITUTION TO THE HEME BINDING MICRO-MYOGLOBIN

M. Haeubl, M. Kriegl, R. Goessler, W. Schoefberger, N. Müller

*Institute of Organic Chemistry, Johannes Kepler University Linz,  
Altenberger Str. 69 4040 Linz Austria  
E-mail: haeubl@inode.at*

Apo- $\mu$ -Myoglobin is a heme binding 78-amino-acids fragment of sperm whale myoglobin. The unlabeled and  $^{15}\text{N}$ -labeled apo- $\mu$ -myoglobin were expressed in *E. coli* BL21( DE3)-cells containing the plasmid pSSB1 [1, 2]. One of the biggest problems after the protein expression was precipitation of this protein. During our investigation we found proper conditions where apo- $\mu$ -myoglobin shows its highest solubility.

We used CD-measurements, UV-vis spectra, COSY, TOCSY and NOESY NMR measurements for the unlabeled apo- $\mu$ -myoglobin. While the  $^{15}\text{N}$  labeled compound was investigated by preliminary  $^{15}\text{N}$ -HSQC NMR to verify the protein's folding conditions.

In the next step we prepared unlabeled holo- $\mu$ -myoglobin. This was done in a reconstitution process where the apo- $\mu$ -myoglobin was mixed with an equimolar amount of heme which binds to  $\mu$ -myoglobin and changes the protein's folding conditions. Due to this change the surface of  $\mu$ -myoglobin gets more hydrophobic and the pro-

tein is more likely to precipitate. We found suitable conditions for the reconstitution of apo- $\mu$ -myoglobin and CD-measurements of the holo- $\mu$ -myoglobin suggested a change in the folding state of holo- $\mu$ -myoglobin. This change of folding is also visible in the UV-vis spectra where the absorption maximum shifts from 275 nm to 412 nm. [1] The heme itself absorbs at 385 nm. Unfortunately the holo- $\mu$ -myoglobin is very likely to precipitate in the chosen buffer. Therefore any attempts to concentrate the reconstituted protein for NMR measurements failed. To keep the protein soluble we changed the buffer conditions and the protein's solubility increased significantly. This route seemed to be a very promising way to achieve the necessary concentrations for NMR experiments.

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P2

### HUMAN PROTEIN CD69 STUDIED BY COMBINATION OF VIBRATIONAL SPECTROSCOPY AND MOLECULAR MODELING

K. Hofbauerová<sup>1,2</sup>, V. Kopecký Jr.<sup>2</sup>, R. Ettrich<sup>3</sup>, O. Vaněk<sup>4</sup>, J. Pavlíček<sup>4</sup> and K. Bezouška<sup>1,4</sup>

<sup>1</sup>*Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Prague 4, CZ-14220, Czech Republic; hofbauer@biomed.cas.cz*

<sup>2</sup>*Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, Prague 2, CZ-12116, Czech Republic*

<sup>3</sup>*Laboratory of High Performance Computing, Institute of Physical Biology of USB and Institute of Systems Biology and Ecology of AS CR, Zámek 136, Nové Hrady, CZ-37333, Czech Republic*

<sup>4</sup>*Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, Prague 2, CZ-12840, Czech Republic*

CD69 (Cluster of Differentiation) is the earliest leukocyte activation antigen playing a pivotal role in the cellular signalling [1, 2]. It belongs to the type II of transmembrane proteins possessing an extracellular C-terminal protein motif related to C-type animal lectins. CD69 is a disulfide-linked homodimer with two constitutively phosphorylated and variously glycosylated chains. It is expressed on the cell surface only 30–60 minutes after stimulation.

To initiate structural and ligand binding studies, we have prepared soluble monomer form of human CD69 protein (Gly<sup>70</sup>-Lys<sup>199</sup>) by recombinant expression of its

extracellular domain, using pRSET B vector, in *Escherichia coli* followed by *in vitro* refolding. To investigate potential of CD69 for ligand binding a large scale of ligands with different stereo-chemical properties was selected – GlcNAc a basic carbohydrate, tri and penta-antennary carbohydrates, and a short peptide with the sequence LELTEGY. The influence of binding of those ligands on the secondary structure of CD69 as well as on local environments of amino acids with aromatic side chains was followed by Raman spectroscopy and Fourier transform infrared spectroscopy (FTIR). Raman and FTIR spectros-

copy are very sensitive methods for investigation of differences between bound and native stage of the protein with respect to its secondary structure content. Moreover, they can provide detail information of certain amino acid residues. Such a big ligands as antennary sugars or peptides can have an influence on protein dynamics, wherefore thermal dynamics of CD69 with and without ligands was investigated by Raman spectroscopy.

The protein homology model, based on template crystal structure of CD69 1E87 [2], was used for identification of ligand binding sites. Refinement was achieved through algorithmic analysis and a minimization was done with the TRIPOS force field in the SYBYL/MAXIMIN2 (Tripos) module. Docking of a calcium ion was explored with the DOCK module of SYBYL. Interactions with ligands were studied using the AUTODOCK program, which is suite for automated docking of flexible ligands to receptors. The binding was investigated by usage of molecular dynamics calculations, which reveals small changes in protein CD69

structure upon ligand binding. Results from theoretical calculations were critically confronted with experimental measurements, whereas good agreement was reached.

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P3

## EVOLUTION OF HALOALKANE DEHALOGENASE PROTEIN FAMILY

E. Chovancová<sup>1</sup>, J. Kosinski<sup>2</sup>, J. M. Bujnicki<sup>2</sup> and J. Damborský<sup>1</sup>

<sup>1</sup>*Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, CZ*

<sup>2</sup>*Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, ul. Ks. Trojdena 4, 02-109 Warsaw, PL*

*E-mail: akllupe@chemi.muni.cz*

Enzymes from the haloalkane dehalogenase protein family (EC 3.8.1.5) play an important role in bioremediation processes for their capability to hydrolyze haloorganic compounds. Currently, this family includes 14 experimentally characterized enzymes with proven dehalogenation activity. In addition to them, many putative members of this family can be found in sequence databases. In this study, we have used phylogenetic approach to assess the origin and evolution of haloalkane dehalogenases. Knowledge of the evolutionary history of haloalkane dehalogenases and related protein families should provide basis to understand the evolution of enzymatic activities and structure-function relationships.

Over 3000 protein sequences, including haloalkane dehalogenases and their homologs, were identified through PSI-BLAST database searches [1]. Obtained sequences were clustered using the program CLANS [2]. Sequences from the cluster of haloalkane dehalogenases were aligned and used for phylogenetic reconstructions by maximum-likelihood [3] and neighbor-joining [4] methods. Various evolutionary models with different parameters were tested. For rooting of resulting trees, three alternative outgroups were used. Phylogenetic trees from all analyses were compared in terms of tree topology and placement of the root. Statistical techniques including four-cluster likelihood mapping [5] were further used to test phylogenetic hypotheses.

Phylogenetic analysis of haloalkane dehalogenases indicated that the members of this family comprise three subfamilies designated HAD-I, HAD-II and HAD-III.

Most of biochemically characterized haloalkane dehalogenases (9 of 14) belong to the subfamily HAD-II. Calculations performed under various conditions did not show significant differences in the results. Sister-group relationship was suggested for subfamilies HAD-I and HAD-III, while HAD-II subfamily appeared to be more distantly related. Preferential grouping of HAD-I and HAD-III subfamilies was indicated also by four-cluster likelihood mapping. Based on the results, hypothesis about evolutionary history of haloalkane dehalogenases was proposed. Results of the analysis enabled identification of new family members and subsequent phylogenetic classification of the whole family. Furthermore, evolutionary studies of haloalkane dehalogenases will be useful for determination of conserved regions. Altogether, results will lead to improved theoretical predictions of functional, biochemical and structural properties of novel proteins. Putative haloalkane dehalogenases with potentially interesting properties will be cloned and biochemically characterized. This may lead to acquisition of proteins with novel characteristics suitable for practical applications.

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P4

## COMPUTER-ASSISTED DESIGN OF FLUORESCENT PROBES FOR SOLVENT RELAXATION EXPERIMENTS

P. Jeřábek<sup>1</sup>, J. Brezovský<sup>1</sup>, M. Hoř<sup>2</sup>, J. Damborský<sup>1</sup>

<sup>1</sup>Loschmidt Laboratories, Masaryk University, Brno, Czech Republic

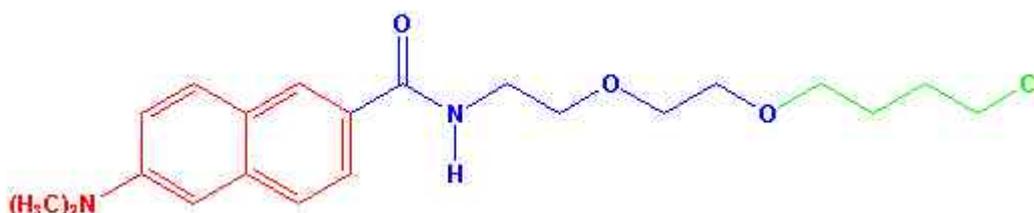
<sup>2</sup>J. Heyrovsky Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

The presence and dynamics of solvent molecules may have considerable impact on binding affinities and catalytic rates of enzymes. Solvent relaxation experiments can be used to study solvent dynamics to molecular level. A fluorescent probe with good affinity for the active site of a protein is required for these experiments. Objective of this study was to design probe molecules with appropriate spectral and binding properties.

Dehalogenation reaction proceeds in the three reactions steps – nucleophilic substitution, nucleophilic addition and elimination, during which a substrate is converted to a product. A mutant form of the enzyme with inhibited nucleophilic addition preventing a probe leaving from the active site should be constructed and used for the experiments. This will make possible to study the solvent motion around the probe with fluorescence spectroscopy. Three haloalkane dehalogenase enzymes were selected and genetically modified LinB from bacterium *Sphingomonas paucimobilis* UT26, DhaA from bacterium *Rhodococcus sp.* NCIMB13064 and DbjA from bacterium *Bradyrhizo-*

*bium japonicum* USDA110. Crystal structures of these enzymes were used to construct single-point mutants of a catalytic histidine to phenylalanine.

Molecular docking was then used to design probes with appropriate properties. The main task of this study was to find an appropriate length of reactive linker (Figure 1), which is responsible for location of fluorescent part of the probe inside the enzyme. This part of a probe can be positioned in the active site, in the tunnel joining the active site with protein surface or can stick out from the enzyme to a bulk solvent. According to position of fluorescent probe one can potentially study solvent dynamics in different parts of an enzyme. Probes with different length of reactive linkers were systematically modelled into individual enzymes. The prodan molecule was selected as a fluorescent part of probes (Figure 1). Several fluorescent probes with different position of prodan part inside the enzyme active site were designed. These probes will be synthesised and tested experimentally.



**Figure 1.** Structural formula of a fluorescent probe. The fluorescent part of molecule is in red, the reactive linker is in blue and the substrate part is in green.

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## MYCOBACTERIA AS A SOURCE OF HALOALKANE DEHALOGENASES: IDENTIFICATION OF THE CATALYTIC PENTAD WITHIN THE STRUCTURE OF DHMA FROM *Mycobacterium avium* N85

Andrea Jesenská<sup>1,2</sup>, Martina Pavlová<sup>1</sup>, Martin Klvaňa<sup>1</sup>, Radka Chaloupková<sup>1</sup>, Marta Monincová<sup>1</sup>, Takashi Sato<sup>3</sup>, Masataka Tsuda<sup>3</sup>, Yuji Nagata<sup>3</sup>, Zbyněk Prokop<sup>1,2</sup> and Jiří Damborský<sup>1</sup>

<sup>1</sup>Loschmidt Lab., Fac. of Science, Masaryk University, Kamenice 5/A4, Brno, 611 37, Czech Rep.

<sup>2</sup>National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5/A4, Brno, 611 37, Czech Republic

<sup>3</sup>Department of Environmental Life Science, Graduate School of Life Sciences, Tohoku University, Katahira, Sendai 980-8577, Japan

Haloalkane dehalogenases belonging to /-hydrolase superfamily are microbial enzymes that catalyze hydrolytic cleavage of carbon-halogen bond. This reaction plays a key role in biodegradation of halogenated aliphatic compounds. Haloalkane dehalogenases act on broad range of halogenated aliphatic compounds. These enzymes were isolated mainly from bacteria colonizing contaminated environment.

Comparison of known haloalkane dehalogenases with sequences provided by genomic projects showed that mycobacteria belong to valuable source of new putative haloalkane dehalogenases. Five haloalkane dehalogenases from saprophytic and tissue-colonizing mycobacteria were cloned: *dhmA* from *Mycobacterium avium* N85 [1], *dmsA* from *Mycobacterium smegmatis* CCM4622, and *dmbA*, *dmbB* [2] and *dmbC*, all from *Mycobacterium bovis* 5033/66. The proteins encoded by the genes were expressed using different expression systems and purified to homogeneity by metal affinity chromatography. The biochemical characterization of mycobacterial haloalkane dehalogenases showed that they differ in pH, temperature optimum, melting temperature and substrate specificity.

Haloalkane dehalogenase DhmA is the best characterized from the structural point view. Catalytic pentad consists of catalytic triad and two halide-stabilizing amino acid residues. The catalytic triad D123, H279 and D250 was deduced from sequence comparison. Amino acid W124 was identified as the first halide-stabilizing residue. The second halide-stabilizing residue W164 was predicted based on homology modeling. Five single-point mutants D123A, W124L, W164L, D250A and H279A were constructed, expressed in *Rhodococcus erythropolis* IAM1399 and purified to homogeneity. None of the DhmA mutant showed dehalogenating activity confirming their essential role in catalysis. A reaction mechanism was proposed for DhmA and compared with the mechanism of other family members.

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P6

## BEHAVIOR OF THE HUMAN $\alpha_1$ -ACID GLYCOPROTEIN IN WATER-LOW ALIPHATIC ALCOHOL SYSTEMS

L. Maršálková<sup>1</sup>, V. Kopecký Jr.<sup>2</sup>, V. Karpenko<sup>1</sup>

<sup>1</sup>Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 6, CZ-12843 Prague 2, Czech Republic

<sup>2</sup>Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, CZ-12116 Prague 2, Czech Republic; E-mail: kopecky@karlov.mff.cuni.cz

The  $\alpha_1$ -acid glycoprotein (AGP), also known as orosomucoid, is a 41-kDa single polypeptide formed of 183 amino acids with 21 possible substitutions in one polypeptide chain linked by two disulfide bridges. Carbohydrate moiety is slightly over 40% of its total molecular weight. It is formed of five branched carbohydrate units linked to Asp of the peptide chain by the *N*-glycosidic bond and terminated by sialic acid residues [1]. Concerning useful chromophore groups there are 3 Trp, 12 Tyr, and up to

12 Phe residues in AGP molecule. The native structure of AGP is characterized by low  $\alpha$ -helix content and is dominated by  $\beta$ -sheets [2]. A model of three-dimensional structure of AGP has been published recently [3], which have been proposed by an approach that combines molecular modeling and vibrational spectroscopy. It shows that AGP folds as a highly symmetrical all- $\beta$  protein dominated by a single eight-stranded antiparallel  $\beta$ -sheet. AGP is a glyco-



protein that occurs physiologically in human blood serum. It is known that AGP plays a role under inflammatory or other pathological conditions and is able to bind basic drugs and certain steroid hormones such as progesterone. However, its biological function and complete 3D structure remains unknown.

AGP has been studied over the whole pH range in the aqueous solutions. Behavior in the presence of alcohol has been examined only in the basic region [4]. We focused our attention on acid pH range, because isoelectric point of AGP varies between 1.9 and 2.7 depending on the buffer used for its determination. It is supposed that presence of alcohol in the system influences both intra- and intermolecular interactions of this protein. Alcohols may cause changes in the structure of water and, consequently, the hydration shell of AGP molecule is likely to be disturbed. Furthermore, alcohols decrease permittivity of the solvent, and thus the electrostatic interactions in the system.

The aim of the present work was to examine the effect of four aliphatic alcohols – methanol, ethanol, 1-propanol and 2-propanol, on the structure of AGP molecule in the acid region. Weight fraction of all alcohols was 0.10; the experiments have been performed in the pH region 2–7. Behavior of AGP in the presence of alcohols has been studied by means of infrared spectroscopy, UV-VIS absorption spectroscopy and fluorescence spectroscopy. Spectral sets of each used spectroscopic method used (at desired pH or in presence of an aliphatic alcohol) have been subsequently

examined by principal component analysis. The two-dimensional (2D) correlation spectroscopy was applied as well as a new method of a multivariate statistics which provides the specific order of the spectral intensity changes taking place during the measurement on the value of variable affecting the spectra. Moreover, heterospectral 2D correlation spectroscopy enables joint data evaluations of spectral sets measured by different spectroscopic methods at the same conditions. Finally, it has been demonstrated that the presence of alcohols in the most acid part of the studied pH region has significantly influenced AGP structure, particularly it has led to decrease of  $\beta$ -sheet content.

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P7

## CRYSTALS OF PHOTOSYSTEM II CORE COMPLEX FROM *Pisum sativum*

Ivana Kutá Smatanová<sup>1,2</sup>, José A. Gavira<sup>3</sup>, Pavlína Řezáčová<sup>4</sup>, František Vácha<sup>1,5</sup>, and Juan M. Garcia-Ruiz<sup>3</sup>

<sup>1</sup>*Institute of Physical Biology USB CB, Zámek 136, 373 33 Nové Hrady, Czech Republic*

<sup>2</sup>*Inst. of Systems Biology and Ecology AS CR Zámek 136, 373 33 Nové Hrady, Czech Republic*

<sup>3</sup>*Laboratorio de Estudios Cristalografico, Edificio BIC-Granada, Avda. de la Innovación 1, P.T. Ciencias de la Salud, 18100-Armilla, Granada, Spain*

<sup>4</sup>*Dep. Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-8816*

<sup>5</sup>*Institute of Plant Molecular Biology AS CR, Branišovská 31, 370 05 České Budějovice, Czech Republic*

The photosystem II (PSII), the system where oxygenic photosynthesis in plants, algae and cyanobacteria is initiated, is a homodimeric multisubunit protein-cofactor complex consisting of membrane in-lying subunits, hydrophilic peripheral subunits and large number of cofactors as chlorophylls, pheophytins, carotenoids, plastoquinones, iron and manganese, which together trap, transfer and modulate a sunlight and drive the unique photoinduced oxidation of water to atmospheric oxygen. Catalytic mechanism of PSII has been studied using a wide range of approaches [1-3], but particular molecular details of water oxidation catalyzed by the oxygen evolving center (OEC) remains unclear. Crystallographic studies of cyanobacterial OEC PSII from thermophilic cyanobacterium have provided several medium-resolution structures from resolution 3.8 Å to 3.2 Å [4-6]. Results of mentioned studies

have given the first description of the structure of PSII, but present models are not absolutely complete as yet.

Finding suitable crystallization condition is the main problem to solve a protein structure by X-ray diffraction techniques. The current crystallization strategies are mainly based on the screening upon previously successful chemical cocktails and on classical crystallization techniques based on evaporation. That strategy has been found partially successful for soluble globular proteins. However, membrane protein is a particular case for which the success rate applying commercial crystallization screens is much lower than for soluble proteins. The fact that membrane proteins are often unstable, highly temperature and light sensitive together with their complicated composition are responsible for difficult crystal growing and solving their structure.

Crystallization experiments of monomeric and dimeric photosystem II core complex (OEC PSII) from *Pisum sativum* have been already described [7]. Here we report following experiments aimed at obtaining better-quality crystals suitable for diffraction analysis. We have studied the influence of additives (MgCl<sub>2</sub>, MgSO<sub>4</sub>, MnCl<sub>2</sub>, MnSO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, CdSO<sub>4</sub>) and detergents (DM, LDAO, CAPS and Zwittergent 3-12) on the crystallization behavior of protein complex. We expect to obtain typical photosystem II core complex crystals for initial crystallographic characterization.

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P8

## PHOTOSYNTHETIC REACTION CENTRE AND CHLOROSOMAL PIGMENTS. A COMPUTATIONAL STUDY

M. Kutý<sup>1</sup>, P. Palenčár and J. Pšenčík<sup>3</sup>

<sup>1</sup>*Institute of Physical Biology, University of South Bohemia, Zámek 136, 37333 Nové Hradky, Czech Republic*

<sup>2</sup>*Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, Zámek 136, 37333 Nové Hradky, Czech Republic*

<sup>3</sup>*Faculty of Mathematics and Physics, Charles University, Ke Karlovu 3, 121 16 Prague, Czech Republic*

Photosynthetic pigments are subject of wide variety of studies, in particular electronic structures of the ground and excited state. Molecular modelling method such as molecular dynamics coupled with quantum chemistry is a powerful tool for understanding and interpreting optical spectra experiments [1]. Quantum chemical calculations have been applied to elucidate absorption and CD spectra of chlorophyll *a*, pheophytine *a* and bacteriochlorophyll *c* monomers and multimers and their excitonic interaction. In this study we examine excited states by: a) semiempirical ZINDO/S calculations, b) configuration interaction (CI) and time dependent method at *ab initio* HF and density functional approximation with Pople basis set 6-31G (including diffusion and polarization functions) and c) empirical point-dipole calculations (diagonalization of Frenkel Hamiltonian). All mentioned calculations were performed

on actual X-ray molecular structures of algae photosystem II reaction centre and on a theoretical model of chlorosomal bacteriochlorophyll "c". The spectral effects of the functional groups and pigments orientation were interpreted.

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P9

## OVEREXPRESSION, PURIFICATION AND CRYSTALLIZATION ATTEMPTS OF THE RECOMBINANT R SUBUNIT *EcoAI*

M. Lapkouski, E. Jindrová and I. Kutá Smatanová

*Institute of Physical Biology, University of South Bohemia, Zamek 136, CZ-37333 Nove Hradky, Czech Republic, Email lapkouski@greentech.cz*

Type I DNA restriction enzymes are large molecular machines comprising DNA methyltransferase, ATPase, DNA translocase and endonuclease activities, essential for the antiviral defence in bacterial cells. The ATPase, DNA translocase and endonuclease activities are specified by the restriction (R) subunit of the enzyme. Our work is focused mainly on revealing structural features of the R subunit of the endonuclease *EcoAI* from *Escherichia coli*. Having optimized purification methods, crystallization trials were performed as a step for farther structural characterization of the enzyme.

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P10

## STABILIZATION OF ANTIBODY STRUCTURE UPON BINDING EPITOPE PEPTIDE: X-RAY, MICROCALORIMETRY AND MOLECULAR DYNAMICS STUDY

P. Mader<sup>1</sup>, J. Brynda<sup>1</sup>, V. Král<sup>1</sup>, P. Řezáčová<sup>1,2</sup>, R. Štouračová<sup>1,3</sup>, M. Fábry<sup>1</sup>, J. Závada<sup>1</sup>, Z. Zavadová<sup>1</sup>, L. Rulíšek<sup>4</sup>, M. Kožíšek<sup>4</sup>, J. Sedláček<sup>1</sup>

<sup>1</sup>*Institute of Molecular Genetics, Flemingovo nám. 2, CZ-166 37 Praha 6*

<sup>2</sup>*UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, Texas 75390*

<sup>3</sup>*University of Colorado at Denver, Health Sciences Center, 4200 E. Ninth Ave. Denver, CO 80262*

<sup>4</sup>*Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, CZ-166 37 Praha 6  
E-mail: mader@img.cas.cz*

Among fourteen human carbonic anhydrases, only the CA IX isoform is strongly associated with certain types of cancer. A unique structural feature of CA IX is its extra component - the proteoglycan-like (PG) segment, located at the amino terminus of the molecule. Conceivably, this segment could be of relevance for oncogenesis, therefore it deserves a more detailed investigation.

The PG amino acid sequence [Q16790; gi:5915865] comprises 59 residues:

**SSGEDDPLGEEDLPSEEDSPREEDPPGEEDLPGEEDLPGEEDLP**  
**DLPGEEDLP**EVKPKSEEEGSLKLE

A remarkable feature of the PG segment of the CA IX molecule is a high content of dicarboxylic amino acids (27 D + E out of total 59 residues) and a low content of basic ones (4 R + K). Most of the dicarboxylic amino acids are grouped in four identical repeats of the motif GEEDLP (bold) and in its three modified versions. The epitope pep-

tide used in this study (underlined) seems to be an adequate structural representation of the protein antigen since predictions show lack of any secondary structure in the PG domain. Abnormal expression of CA IX in various commonly occurring carcinomas suggests its involvement in oncogenic pathways. CA IX is also a cell adhesion molecule (CAM) that can mediate attachment of cells to non-adhesive solid support [1]. For a secondary structure-lacking, flexible peptide it might be intuitively expected that such ligand would adopt a complementary shape and undergo stabilization in the complex with its cognate antibody: this would be accompanied with a loss of the conformational freedom and with an unfavorable entropy contribution, measurable with microcalorimetry methods. We show here that upon the epitope peptide binding a substantial structural re-arrangement occurs also in one of the antibody hypervariable loops and that the accompanying local stabilization can be traced in comparisons of the crystal structures of the free and complexed antibody. Somewhat

unexpectedly, all substantial 3D structural transitions occur in the hypervariable loops other than those that form counterparts of the epitope dicarboxylic amino acid residues and that provide for major enthalpy contributions.

P11

## TEMPERATURE DEPENDENCE OF HUMAN $\alpha_1$ -ACID GLYCOPROTEIN BEHAVIOR INDUCED BY MIXED SOLVENTS AND ACID PH

L. Maršáľková<sup>1</sup>, V. Kopecký Jr.<sup>2</sup>, V. Karpenko<sup>1</sup>

<sup>1</sup>Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Albertov 6, CZ-12843 Prague 2, Czech Republic; E-mail: marsalko@natur.cuni.cz

<sup>2</sup>Institute of Physics, Faculty of Mathematics and Physics, Charles University, Ke Karlovu 5, CZ-12116 Prague 2, Czech Republic

Orosomucoid is one of the original names of  $\alpha_1$ -acid glycoprotein (AGP throughout this paper), which is a protein from the  $\alpha_1$ -globuline fraction of human blood serum [1] classified among the superfamily of lipocalins. Its molecule contains 183 amino acids with 21 possible substitutions in one polypeptide chain linked by two disulfide bonds. The content of carbohydrate moiety is slightly over 40% of the total molecular weight [1] of 41 kDa of AGP. Five branched carbohydrate units terminated by sialic acid residues are linked to asparagine residues of the peptide chain by the *N*-glycosidic bond. The isoelectric point of AGP has been found to vary between pH 1.8 and 2.7, depending on the buffer chosen. The native structure of AGP is characterized by a low  $\alpha$ -helix and a high  $\beta$ -sheet [2] content. Although AGP has been studied for over fifty years and successfully crystallized already in 1959, all attempts at its X-ray analysis have failed; there is only a 3D computer model [3] of the peptide part of the AGP molecule based on molecular and homology modeling. AGP is a glycoprotein occurring naturally in human blood serum; it is well-known that its blood level increases under some pathological conditions, but the exact role of this protein is not entirely clear.

This glycoprotein has been studied in our laboratory in the basic pH range in both aqueous and mixed solvents, whereas in the acid region the investigation has been done only in aqueous solution [4]. We have chosen the acid pH region for our study because of low isoelectric point of AGP; stability of protein molecules in the vicinity of this pH is of particular interest. As mixed solvent mixtures water – low aliphatic alcohol have been selected. It can be assumed that alcohols exert many-sided effect on protein molecule as well as on the solvent, water. Decrease in permittivity of a solution due to the alcohol results in enhancing of all electrostatic interactions in the system, not only of inter- and intramolecular, but it also concerns the binding of inorganic ions present in the solution. Not less important is the effect of alcohol on the structure of water and on the hydration shell of the protein molecule. With current knowledge of water structure, this effect cannot be quantified.

We have been interested in figuring out the temperature changes of AGP molecule in presence of the two lowest

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aliphatic alcohols – methanol, and ethanol in the acid region. Weight fraction of alcohols was 0.20 over whole pH range studied (pH 2–6) except for pH 3. At this pH it was necessary to lower weight fraction of methanol to 0.01, and ethanol to 0.10, respectively. Temperature measurements consisted of three steps: heating of the sample from 25 °C to 80 °C, cooling it back on 25 °C, and heat it again on 80 °C. The behavior of AGP has been studied using UV-VIS absorption spectroscopy, fluorescence spectroscopy and spectroscopy of circular dichroism. Spectral sets of each spectroscopic method used (at desired pH or at an aliphatic alcohol) have been examined by principle component analysis. Concerning statistical methods, two-dimensional (2D) correlation spectroscopy was applied as well as a new method of a multivariate statistics which provides the specific order of the spectral intensity changes taking place during the measurement on the value of variable affecting the spectra. Moreover, heterospectral 2D correlation spectroscopy enables joint data evaluations of spectral sets measured by different spectroscopic methods at the same conditions. Finally, it has been demonstrated that in presence of both studied alcohols AGP has the lowest solubility in the narrow region around pH 3. Cyclic temperature measurement made it possible to follow course of structural changes in the molecule from the point of view of their reversibility. Our experiments have shown pronounced influence of both temperature and pH on the thermal stability of AGP molecule in the aqueous solution of alcohols.

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P12

## THEORETICAL INVESTIGATION OF THE EFFECTS OF MUTATIONS OF THE CHARGED AMINO ACIDS ON THE DISSOCIATION AND RATE CONSTANTS FOR THE DNA REPLICATION BY HUMAN DNA POLYMERASE

V. Martínek<sup>1,2</sup>, J. Florián<sup>2</sup>

<sup>1</sup>Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

<sup>2</sup>Department of Chemistry, Loyola University Chicago, Chicago, IL 60626, USA  
E-mail: vacmar@natur.cuni.cz

DNA polymerase ( $\text{pol } \beta$ ), the most studied member of the X family of DNA polymerases, is known to be an important part of the eukaryotic base-excision repair system. Furthermore, mutations of this enzyme are associated with many colon and lung tumors and esophageal cancer (1).  $\text{pol } \beta$  is a relatively small (39 kDa) template-dependent DNA polymerase without proofreading activity, which makes it an attractive system for *in vitro* and *in silico* studies of the replication fidelity.

We have performed correlation studies for human  $\text{pol } \beta$  (WT) and six  $\text{pol } \beta$  mutants (R183A, Y271A, D276V, K280G, R283A, E295A). The fidelity related experimental kinetic data, specifically the equilibrium dissociation constant ( $K_D$ ) and the rate of incorporation ( $k_{\text{pol}}$ ), are available for all mutants employed in this study. dNTP binding free energy ( $G_{\text{bind}}$ ), reaction activation energy ( $g_{\text{cat}}^\ddagger$ ) and an apparent reaction activation energy ( $g_{\text{enz}}^\ddagger$ ) derived from  $K_D$ ,  $k_{\text{pol}}$  and  $k_{\text{pol}}/K_D$  were correlated with several structural and energetic parameters calculated from molecular dynamics (MD) trajectories. For each  $\text{pol } \beta$  mutant, a 4 ns MD simulation was carried out in a solvent sphere of 25 Å radius using Amber-95 force field.  $G_{\text{bind}}$  for the dCTP bound to polymerase-DNA complex containing the correct templating nucleotide (G) was computed using the linear interaction energy and linear response approximation methods. In addition, population analysis of the set of 12 distance parameters including the near-attack configuration of the phosphor-ester forming bond was correlated to

the experimental data. The statistically significant correlation was observed between experimental  $g_{\text{enz}}^\ddagger$ ,  $g_{\text{cat}}^\ddagger$  and  $G_{\text{bind}}$  versus the population ratio of conformations forming H-bond (ND2 of Asn279 and O2 of the incoming dCTP base). This finding supports hypothesis that Asn279 residue play a significant role not only in the substrate binding stabilization (corr. coefficient 0.72), but is also as factor influencing the reaction activation energy (corr. coefficient 0.77). These two effects results in high correlation coefficient (0.92) between the H-bond stability (connecting Asp279 with dCTP) and apparent reaction activation energy. Minor relationship to the  $g_{\text{enz}}^\ddagger$  could be also attributed to the catalytic Mg - OD2 (Asp256) coordination bond stability showing correlation coefficient 0.74.

The presented study attributes significant role in the correct (Watson-Crick) nucleotide incorporation reaction to the interaction with Asn279 and with lower extend Asp256. However, in order to be able to fully evaluate the role of these residues in  $\text{pol } \beta$  fidelity the calculations of the mutation effects on the insertion of incorrect incoming deoxynucleotides should be performed.

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P13

## HIGH-THROUGHPUT CHARACTERIZATION OF ENZYMES FROM GENOMIC AND PROTEOMIC PROJECTS – MULTIVARIATE STATISTICAL APPROACH

M. Monincová<sup>1</sup>, A. Jesenská<sup>1</sup>, R. Chaloupková<sup>1</sup>, Y. Sato<sup>2</sup>, Z. Prokop<sup>1</sup> and J. Damborský<sup>1</sup>

<sup>1</sup>Loschmidt Laboratories, Faculty of Science, Masaryk University, Kamenice 5/A4, 625 00 Brno, Czech Republic

<sup>2</sup>Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Sendai 980-8577, Japan

High-throughput genomic and proteomic methods identify a large number of novel enzymes which require systematic characterization. Here, we introduce a novel approach for characterization of enzymes with broad substrate specificity using multivariate statistics. The approach employs multivariate statistical method, Principle Component Anal-

ysis [1], for selection of sufficiently large set of substrates from initial pull of chemicals respecting maximum variability in physical-chemical properties [2]. Quick and reliable enzymatic assay follows the selection and produces an activity data of particular proteins with selected substrates. Third step is the application of Principal Component Anal-

ysis on enzyme activity data matrix to describe the differences in their substrate specificities. The obtained dataset, complemented with physical-chemical properties of halogenated hydrocarbons tested, can be further submitted to Quantitative Structure-Activity Relationships analysis [3].

The concept has been verified with bacterial enzymes haloalkane dehalogenases. Members of this enzyme family cleave carbon-halogen bond in halogenated hydrocarbons and can be used in bioremediation, industrial biocatalysis or as biosensors. A large number of putative enzymes has been identified in genomic sequencing projects. Principle Component Analysis was used for identification of novel enzymes with promising biotechnological properties. Quantitative Structure-Activity Relationships analysis brought detailed view into explanation of activity or inac-

tivity of 30 substrates selected from the set of 194 different halogenated compounds. Such information is helpful in process of rational design and improvement of haloalkane dehalogenases for biotechnological applications.

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**P14****STRUCTURAL STUDY ON C-TERMINUS OF THE VANILLOID RECEPTOR TRPV1****V. Mráziková<sup>1</sup>, E. Jindrová<sup>1</sup>, V. Vlachová<sup>2</sup>, R. Ettrich<sup>1</sup>, J. Teisinger<sup>2</sup>**

<sup>1</sup>Laboratory of High Performance Computing, Institute of Systems Biology and Ecology ASCR, Institute of Physical Biology USB Zámek 136, CZ-373 33 Nové Hradky, Czech Republic.

<sup>2</sup>Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Praha 4  
Email: mrazikova@greentech.cz

The vanilloid receptor (TRPV1) is a member of TRP channel family and has function as a multimodal signal transducer of noxious stimuli in the mammalian somatosensory system [1]. The TRPV1 is consisted of six transmembrane-spanning domains with a pore forming region between fifth and sixth domains, and cytoplasmically located C- and N-terminal regions. Although structural and functional studies have been done [2, 3], the possible contributions of terminal regions to vanilloid receptor function remain elusive. To determine structure and functional properties of the cytoplasmically located tail, the DNA fragment encoding the C-terminus was cloned to the expression vector *pGEX-4T-1* and transformed to *E. coli* strain (*BL 21 (DE3)*). Protein was expressed as fusion protein with the 26 kDa glutathione S-transferase (GST) and was purified by affinity and ion-exchange chromatography. Because of the denaturation of protein and very weak expression output we decided for subcloning to another expression vector *pET 32a (+)*. The pET system is developed for expression of difficult soluble protein. Target genes were cloned in the *pET 32a (+)* under control of strong bacteriophage T7 transcription [4], in fusion with genes encoded His-tag S-tag hook and purified by using affinity chromatography. Purified protein will be used for measur-

ing by vibrational spectroscopy. Experimental results will be combined with homology and energetic modeling techniques and we will propose a three-dimensional structure of the C-terminus.

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P15

## NMR STUDY OF PROTEIN CD69

Monika Nálezková, Lukáš Gorčík, Lukáš Židek and Vladimír Sklenář

National Centre for Biomolecular Research, Faculty of Science, Masaryk University in Brno, Czech Republic

The lectin-type proteins, like CD69, are transmembrane, disulphide-linked glycoproteins of natural killer (NK) cells. NK cells play an important role in the immune response to cell-surface displayed antigens, such as those found on virus infected cells, tumor cells, etc. Several oligosaccharides were suggested to be natural ligands of CD69 receptor and NMR method was found convenient for observation of CD69 saccharide interaction.

NMR is noninvasive method, able to observe molecules in environment close to physiological conditions. Unfortunately, NMR is not very sensible and big amount of sample (300 l of 0.2mM at least), usually enriched by nuclei  $^{15}\text{N}$  and/or  $^{13}\text{C}$ , is needed. Preparation of well folded highly concentrated protein sample is thus crucial. At the beginning of this study, the construct of approximately 130 amino acid long extracellular part of human CD69 was prepared by the group of Karel Bezouška, Department of Biochemistry, Faculty of Science, Prague. NMR measurement of the construct showed that protein is totally unfolded. A construct of homologue of rat CD69 was then delivered. Basic 2D NMR experiment found partially structured protein.

In summer 2005, the problem with folding of human CD69 was solved by our collaborators and experi-

ments aimed at ligand binding were started. As binding of saccharides to CD69 receptor is supposed to be dependent on presence of  $\text{Ca}^{2+}$ , titration experiments with  $\text{CaCl}_2$  were performed, followed by titration with GlcNAc and pentaantennary oligosaccharide. It was possible to observe several small changes of chemical shift in NMR spectra correlating chemical shifts of hydrogen and nitrogen. In order to study protein-ligand interactions in more details it was decided to prepare double ( $^{15}\text{N}$ ,  $^{13}\text{C}$ ) labelled sample, and to assign side chain nuclei of the protein. The double-labelled sample has been obtained recently and assignment is currently in progress.

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P16

## PREPARATION OF ANTENNARY OLIGOSACCHARIDE LIGANDS FROM OVOMUCOID AND THEIR BINDING TO CD69 RECEPTOR

M. Navrátil<sup>1</sup>, P. Pompach<sup>2</sup>, K. Bezouška<sup>1,2</sup>

<sup>1</sup>Dept. of Biochemistry, Faculty of Science, Charles University in Prague, Hlavova 8, CZ-12840 Prague 2

<sup>2</sup>Institute of Microbiology, Academy of Sciences of the Czech Rep., Vídeňská 1083, CZ-14220 Prague 2

Oligosaccharides form a very heterogeneous group of compounds. The ability of their monosaccharide units to bind to each other in many ways predicts them to be very variable in both structure and function. It is well known that saccharide structures play important role in immune system, from direct recognition of pathogenic saccharide structures to protein glycosylation, which can have also modulatory or self-protective function. One group of very interesting oligosaccharide compounds are branched antennary oligosaccharides which are present at the surface of tumour cells and are recognized by C-type lectin-like receptors of NK cells. Immune recognition is highly specific and only small difference in epitope structure may lead to opposite response, exact knowledge of this structure is therefore a necessity. Chemical synthesis [1] of antennary oligosaccharide structures is quite difficult. In contrast, there are glycoproteins that provide defined oligosaccharides structures in sufficient yields. Thus, the isolation of

native structures from natural sources seems to be a more efficient and direct way. In order to research for physiological ligands [2, 3] for C-type lectin-like receptors of NK cells we developed a relatively fast method for preparation of pure and well defined antennary oligosaccharides from hen egg white protein ovomucoid. This procedure involves isolation of ovomucoid by fractional precipitation, followed by its purification on HPLC, enzymatic deglycosylation of ovomucoid and finally purification and separation of cleaved oligosaccharides by HPLC. The purity and identity was verified by MALDI-TOF mass spectrometry. Biochemical binding and inhibitor experiments with CD69 receptor revealed the highest affinity for the pentaantennary oligosaccharide. Oligosaccharides were used for structural studies by means of cocrystallisation with CD69, and examination of CD69-saccharide complexes by MS and NMR titration.

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P17

## PAIN: PROGRAM FOR ANALYSIS OF INTERNAL MOTION AN APPLICATION TO MD SIMULATED MOTIONS OF MAJOR URINARY PROTEIN-I

P. Novák, P. Macek, L. Židek, V. Sklenář

NCBR, Fac. of Science, Masaryk University, Kotlářská 2, Brno, 611 37, shaman@ncbr.chemi.muni.cz

Trajectories from the molecular dynamics (MD) simulations of proteins contain wealth of information. From the MD trajectory not only coordinates of all atoms are available, but also their change in course of time as the molecule is varying its position and conformation. Therefore, motional parameters can be extracted from MD trajectories if the proper tools do exist. For this purpose the program PAIN (Program for Analysis of Internal motioN) was written. Some key features implemented in PAIN up to date are calculation of the : correlation function of the interatomic vectors  
generalized order parameter  
frequency dependent generalized order parameter  
conformation-dependent generalized order parameter  
spatial distribution of the interatomic vector orientations  
histogram of the dihedral angle distribution (including number of modes and distribution parameters estimation)

conformation transitions analysis

Program was tested on and used for an analysis of the MD simulation of the mouse Major Urinary Protein-I in an explicit solvent[1]. The calculated order parameters  $S^2$  for a free- and ligand-bound protein supply evidence that mobility in various regions of MUP-I can be directly related to small conformational changes of the free- and complexed protein resulting from modifications of the hydrogen bonding network.

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P18

## 14-3-3 PROTEIN CHANGES CONFORMATION OF NUCLEAR LOCALIZATION SEQUENCE OF FORKHEAD TRANSCRIPTION FACTOR FOXO4

V. Obšilová<sup>2</sup>, J. Večeř<sup>3</sup>, P. Heřman<sup>3</sup>, A. Pabiánová<sup>1,2</sup>, M. Šulc<sup>4</sup>, J. Teisinger<sup>2</sup>, E. Boura<sup>1,2</sup> and T. Obšil<sup>1,2</sup>

<sup>1</sup>Dept. Physical and Macromolecular Chemistry, Faculty of Science, Charles University, 128 43 Prague

<sup>2</sup>Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague

<sup>3</sup>Institute of Physics, Faculty of Mathematics and Physics, Charles University, 121 16 Prague

<sup>4</sup>Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague

The 14-3-3 proteins are a family of regulatory signaling molecules that interact with other proteins in a phosphorylation-dependent manner. 14-3-3 proteins are thought to play a direct role in the regulation of subcellular localization of FoxO forkhead transcription factors. It has been suggested that the interaction with the 14-3-3 protein affects FoxO binding to the target DNA and interferes with the function of nuclear localization sequence (NLS). Masking or obscuring of NLS could inhibit interaction between

FoxO factors and nuclear importing machinery and thus shift the equilibrium of FoxO localization toward the cytoplasm. According to our best knowledge, there is no experimental evidence showing a direct interaction between the 14-3-3 protein and NLS of FoxO. Therefore, the main goal of this work was to investigate whether the phosphorylation by protein kinase B, the 14-3-3 protein, and DNA binding affect the structure of FoxO4 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. Our results also suggest that the DNA-binding domain of FoxO4 remains relatively mobile while bound to the 14-3-3 protein [1].

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P19

## SPOIIS COMPONENTS OF *Bacillus subtilis* PROGRAMMED CELL DEATH

Pamela Pavelčíková, Patrik Florek, Katarína Muchová, and Imrich Barák

*Institute of Molecular Biology, Slovak Academy of Science, 845 51 Bratislava, Slovakia*

The *spoIIS* locus from *Bacillus subtilis* consists of two genes, *spoIISA* and *spoIISB*. The *spoIISA* gene encodes a 248-residue protein. It contains three transmembrane helices and the last two-thirds of the protein form negatively charged domain, localized in the cytoplasm. The SpoIISB is a small, 56-residue, basic, hydrophilic protein.

The *spoIISB* translation start codon overlaps the *spoIISA* translation stop codon [1], which together with our findings indicates, that these two genes constitute an operon.

The *spoIIS* locus is organized in a similar fashion to the addiction modules, which were found in many prokaryotic organisms and through these systems the programmed cell death is frequently mediated in bacteria [2].

Addiction modules consist of two genes, a labile anti-toxin and a stable toxin. The product of the antitoxic protein antagonizes the toxic effect of the latter [2].

In contrast to *spoIISA* mutant, which efficiently sporulates, a null mutation of *spoIISB* leads to a sporulation block short after formation of asymmetric septum. Disruption of the whole *spoIIS* locus has also no effect on sporulation. When induced during vegetative growth in *B. subtilis*, expression of SpoIISA is toxic and causes cell lysis. So this revealed that SpoIISB protein neutralizes the toxic effect of SpoIISA [1].

In this work we showed that SpoIISA has a toxic effect also in *Escherichia coli* cells, when is present without SpoIISB. SpoIISA is responsible for the cell lysis during vegetative growth. In this system we tested different parts of the SpoIISA protein and it was found, that no just transmembrane or cytosolic domain, but only the whole SpoIISA is required for the toxicity. The effect of SpoIISA

protein is then bactericidal, rather than bacteriostatic. We observed very quick decrease of amount of colony forming units in the cell population carrying sole *spoIISA* gene in comparison with cell population carrying *spoIISA* and *spoIISB* genes.

The aim of our research is to solve the structure of the complex formed by cytosolic part of SpoIISA together with SpoIISB protein. As we found, one copy of each *spoIIS* gene leads to expression a very heterogenic protein complexes caused by an abundance of SpoIISA. Thus, we constructed pET-Duet vector derivative containing one copy of the gene corresponding to His<sub>6</sub>-tag fused cytosolic part of SpoIISA with two copies of intact *spoIISB*. After expression we isolated and purified it using Ni<sup>2+</sup> chelate affinity chromatography gaining stable homogenous protein complex, which was used for crystallization trials. In a few screening conditions we obtained promising crystals.

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P20

## STRUCTURAL STUDY OF *Thermoplasma acidophilum* PROTEIN TA1019 BY NMR SPECTROSCOPY

A. Pavelková, P. Macek, J. Chmelík, L. Židek and V. Sklenář

National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, E-mail: annap@chemi.muni.cz

TA1019 is a protein possibly involved in molybdopterin synthesis. It consists of 107 amino acid residues. Structural study of TA1019 represents a part of proteomic project of prokaryote organism, *Thermoplasma acidophilum*. The recombinant protein TA1019 was prepared at the University of Toronto, Canada, by Adelinda Yee and coworkers. Aggregation and low stability of the sample excluded this project from automatically performed structural investigation.

As the first step in the course of structural determination process, assignment of the measured frequencies to the individual atoms of the protein molecule has been carried out. Most of backbone resonances of the native part of the

protein were assigned from a set of triple resonance experiments including HNCA, HN(CO)CA, HNCACB and CBCA(CO)NH, together with C,N-edited NOESY. Side chain resonances were assigned using the HCCH-TOCSY spectrum.

The secondary structure was predicted based on backbone atom chemical shifts. Inter-proton distances from the NOESY spectra and torsion angles and from the predicted secondary structure served as restraints in molecular dynamics structure calculations. The calculated structure is presented and compared to a structurally related protein (sharing 40% sequence identity).

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## CHARACTERIZATION AND CRYSTALLIZATION OF PROTEINS INVOLVED IN CONTROL OF CELL DIVISION AND CHROMOSOME SEGREGATION IN *Bacillus subtilis*

N. Pavlendová, K. Muchová, I. Barák

Institute of Molecular Biology, Slovak Academy of Sciences, 845 51 Bratislava

Gram-positive bacterium *Bacillus subtilis* is the one of the most commonly studied model organism. Its life cycle represents a complex biological process with a machinery of proteins involved. The mechanisms that ensure the correct positioning of the septum and correct chromosome segregation during both vegetative growth and sporulation are relatively complicated and still intensively studied. DivIVA, MinCD, RacA, Spo0J and Soj are the key proteins involved in control of cell division and in chromosome segregation.

DivIVA is a 19.5 kDa tropomyosin-like  $\alpha$ -helical coiled-coil protein. It assembles *in vitro* into a “doggy-bone”-like oligomers, which can build higher structures like strings, wires and 2D nets [1]. DivIVA is a bifunctional protein involved in both vegetative growth and in sporulation. It localizes to the division site and to the cell poles. For polar targeting of DivIVA are responsible amino acid residues at N-terminal domain. Therefore, mutations at these sites may mimic conformational change that probably occurs and enables DivIVA its dual function [2]. During vegetative growth, DivIVA acts as a controller of the mid-cell division site positioning. DivIVA protein arrests an inhibition complex of two proteins: MinC and MinD at cell poles and thus releases the cell center for septum formation [3]. During sporulation, DivIVA participates in chromosome segregation [4]. It interacts directly or indirectly with RacA, which serves as a “bridge” connecting

*ori* regions of chromosome with cell poles [5]. Besides DivIVA and RacA protein, also Soj, Spo0J are involved in chromosome segregation during sporulation. Spo0J protein localizes around the *ori* region and together with dynamically behaving Soj protein ensures precise anchoring of *ori* regions to opposite cell poles [6, 7].

We have cloned genes *divIVAw*, *divIVAR18C*, *racA*, *minD*, *spo0J* and *soj* into pET vectors for expression and purification of all corresponding proteins. They will be used for crystallization and/or protein-protein interaction assays.

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P22

## MD/DFT STUDY OF ENVIRONMENTAL EFFECTS ON <sup>31</sup>P CHEMICAL SHIFT TENSORS IN B-DNA

**J. Přecechtělová<sup>1</sup>, P. Novák<sup>1</sup>, M. Kaupp<sup>2</sup>, M. L. Munzarová<sup>1</sup> and V. Sklenář<sup>1</sup>**

<sup>1</sup>*National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic*

<sup>2</sup>*Institut für Anorganische Chemie, Universität Würzburg, Am Hubland, D-97074, Germany*

<sup>31</sup>P NMR techniques have become a powerful tool to probe the conformation of the phosphodiester backbone in nucleic acids [1]. A few years ago, a new method based on <sup>31</sup>P chemical shift anisotropy (CSA) was suggested for constraining the orientation of the phosphate groups relative to the molecular alignment tensor [2]. This method, though very helpful in nucleic acid (NA) structure determination, suffers from adopting an assumption that the <sup>31</sup>P CSA tensor is uniform for all backbone phosphates in oligonucleotides. In order to check the justification of such an assumption, we have attempted to perform a combined MD/DFT study of <sup>31</sup>P chemical shift (CS) tensors.

For this purpose we tested three models differing in the size of the sugar-phosphate backbone involved. As the study requires a very precise description of environmental effects, we have also carried out a series of validation calculations to find the optimal strategy for the solvent treatment and the treatment of long-range polarization effects. The tested methodologies included an explicit solvent, the PCM-COSMO model and the charge-field perturbation approach that was used to model water molecules lying beyond the first solvation shell by explicit partial point charges. To reduce the computational costs we took the following steps: 1. the geometries were taken from the molecular dynamics simulation without reoptimizing, 2. the

resolution-of-identity (RI) approximation as implemented in Turbomole5.6 was employed for the wavefunction calculations, and 3. locally dense basis sets were used in NMR calculations performed at the DFT level.

The calculated <sup>31</sup>P chemical shift tensors show a strong sensitivity to the method of the solvent treatment – the combinations of the explicit solvent with the PCM-COSMO model and the point charges, respectively, give qualitatively different chemical shift tensors. In addition, the comparison of the chemical shift tensors for the three models reveal nonintuitive trends. These results will serve as a basis for the careful choice of the appropriate methodology to calculate an average of the <sup>31</sup>P chemical shift tensors over the molecular dynamics trajectory of the oligonucleotide d(CGCGAATTCGCG)<sub>2</sub>.

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P23

## STRUCTURAL STUDY ON THE N-TERMINUS OF THE VANILLOID RECEPTOR TRPV1

**A. Samad<sup>1</sup>, E. Jindrová<sup>1</sup>, V. Vlachová<sup>2</sup>, J. Teisinger<sup>2</sup>, R. Ettrich<sup>1</sup>**

<sup>1</sup>*Laboratory of High Performance Computing, Institute of Systems Biology and Ecology ASCR and Institute of Physical Biology USB, Zámek 136, CZ-373 33 Nové Hrady, Czech Republic.*

<sup>2</sup>*Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Praha 4, Email: samad@greentech.cz*

Transient receptor potential vanilloid receptor-1 (TRPV1) is a cation channel present in sensory nerve endings capable of sensing pain-producing stimuli [1] and believed to play a central role in promoting neurogenic inflammation. To date, mammalian TRPV1 has been cloned and characterized from human [2], rat [3], guinea pig [4], rabbit [5], and mouse [6]. Members of TRPV1 family respond to a variety of selective activators including chemical compounds

such as capsaicin, resiniferatoxin, olvanil and N-oleoyldopamine [7], as well as physiological conditions such as changes in extracellular pH [8], temperature above 42 C and direct phosphorylation via protein kinase C (PKC) [9]. The transient receptor potential vanilloid 1 (TRPV1) is predicted to have six transmembrane (TM) domains and a short, pore-forming hydrophobic stretch between the fifth and sixth TM domains [10]. Like many

other TRP channels, TRPV1 has a long amino terminus containing three ankyrin-repeat domains and a carboxyl terminus containing a TRP domain close to sixth TM [11]. It has been recently shown that calmodulin interacts strongly with the C-terminal domain of TRPV1, similarly, a histidine triad-like protein, PKCi, was identified as interacting partner of the N-terminus of TRPV1 [12] but these findings are still not enough to propose their putative role in TPV1 function. Efforts have been made to unravel the structure of TRPV1 [13,14], but the possible contributions of terminal regions to vanilloid receptor function are still a question. Our aim is to resolve the structure and functional properties of the N-terminal domain of TRPV1. We have cloned a DNA fragment of 775 bp encoding the N-terminal region of TRPV1 in *pET32a (+)* with EcoR V and Hind III restriction sites and its expression under the control of strong bacteriophage T7 transcription [15], in fusion with genes encoding His – tag S – tag hook and purified by using affinity chromatography. Purified protein will be used for measuring by vibrational spectroscopy. Experimental results will be combined with homology and energetic modeling techniques to propose a three – dimensional structure of the N – terminus.

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## OPTIMIZATION OF PROTOCOL FOR IDENTIFYING NOVEL GENES FOR STRUCTURAL STUDY

A. Samad<sup>1,2,3</sup>, A. Bashir<sup>1</sup>, S. A. Malik<sup>2</sup>, M. Ahmad<sup>1</sup>, M. Sajjad<sup>1</sup>, A. Mukbool<sup>1</sup>, S. Moddassir<sup>1</sup>,  
A. Aftab<sup>1</sup>, E. Jindrova<sup>3</sup>, R. Ettrich<sup>3</sup>

<sup>1</sup>National Institute for Biotechnology and Genetic Engineering NIBGE, P.O.Box No 577 Jhang Road  
Faisalabad, Pakistan

<sup>2</sup>Quaid-I-Azam University Islamabad Pakistan

<sup>3</sup>Laboratory of High Performance Computing, Institute of Systems Biology and Ecology ASCR,  
Institute of Physical Biology USB Zámek 136, CZ-373 33 Nové Hradky, Czech Republic  
Email: samad\_aries@yahoo.com

A protocol for identifying novel genes of cotton (*Gossypium hirsutum* L.) fiber through differential display PCR [1,2], visualized by ethidium bromide staining, was optimized. The procedure was used to identify differentially expressed genes at 0 DAP (Day After Pollination) and 10 DAP of developing cotton fiber a single longest cell in plant kingdom [3]. Different populations of total RNA were reversely transcribed using single anchored oligo-dT primers. PCR amplification of relative cDNA was carried out using three single anchored oligo-dT primers (dd1: 5' TTTTTTTTTTTG 3', dd2: 5' TTTTTTTTTTTC 3', dd3: 5' TTTTTTTTTTTTA 3') in combination with five arbitrary primers (random decamers). One percent agarose gel containing ethidium bromide for staining can resolve amplified transcripts over a large range of molecular weights. Several differentially expressed transcripts were successfully identified by this procedure. Isolated genes were used as a probe to screen cDNA library for their full-length genes and cloned into TA cloning vector for DNA sequencing. DNA sequence was blast searched for homology

and translated into protein through translate tools (www.expasy.ch, www.justbio.com). Expanin, Tubulin, Lipid transfer proteins and several uncharacterized fiber proteins (e.g. DQ023530, DQ023531, DQ023532, DQ023533, DQ023534, DQ023535, DQ023536) were cloned in this study [4].

Structural information about these uncharacterized fiber proteins was not determined yet. These uncharacterized fiber proteins will be subcloned in *pET 32a (+)* for expression. Expressed protein will be purified by chromatography and measured through spectroscopy. Experimental results will be analyzed with modeling techniques to propose their three-dimensional structures. The future about these proteins appears bright and tremendously exciting.

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P25

## STRUCTURE AND DYNAMICS OF THE N-TERMINAL LOOP REGION OF PSBQ FROM PHOTOSYSTEM II OF *Spinacia oleracea* STUDIED BY THE MEANS OF MOLECULAR MODELING AND VIBRATIONAL SPECTROSCOPY

J. Ristvejová<sup>1</sup>, V. Kopecký, Jr.<sup>2</sup>, Ž. Sovová<sup>1</sup>, M. Balsera<sup>3</sup>, J. B. Arellano<sup>3</sup>, M. Green<sup>4</sup> and  
R. Ettrich<sup>1</sup>

<sup>1</sup>Laboratory of High Performance Computing, Institute of Physical Biology of USB and Institute of Systems Biology and Ecology of AS CR, Zámek 136, 37333 Nové Hradky, Czech Republic; ettrich@greentech.cz

<sup>2</sup>Inst. of Physics, Faculty of Mathematics and Physics, Charles University, Prague, Czech Rep.

<sup>3</sup>Instituto de Recursos Naturales y Agrobiología (CSIC), Salamanca, Spain

<sup>4</sup>The College of New Jersey, Ewing NJ, United States of America  
E-mail: sovova@greentech.cz

A combination of homology and energetic modeling combined with vibrational spectroscopy was used for the determination of the 20 amino acids loop between two  $\beta$ -sheets of the N-terminus of the PsbQ protein of the oxygen evolving complex bound to Photosystem II of *Spinacia oleracea*. Recombinant PsbQ protein was overexpressed in *Escherichia coli* BL21(DE3)pLysS transformed by JR2592 vector (B96 cells). Restraint-based homology modeling was

used to create a series of initial model structures. Further restraints determined by FTIR and Raman spectroscopy were applied to identify which the initial models were best for molecular dynamics in solution. Initial results from a 20ns molecular dynamics simulation have shown a stable fold of the loop that is in accordance with the spectroscopic data.

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## CORRECTION TO NMR PARAMETERS VIA AVERAGING OVER THE MOLECULAR MOTION

Vladimír Sychrovský and Vladimír Špirko

*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Praha*

NMR parameters are calculated usually for the optimal geometry of given molecule. This point-like geometry however need not necessarily correspond to the statistically significant distribution of atoms/nuclei in the space during the acquisition of NMR signal. When any NMR property involves large variation with respect to local geometry and the amplitudes of respective molecular motions are remarkable, the averaged NMR parameter can differ notably from the parameter calculated for the point-like geometry. Computational approach including anharmonic vibrational corrections is presented on three examples. Vibrational correction of NMR shifts and spin-spin couplings in

LAlanine-LAlanine zwitteranion [1], vibrational corrections of Cross-correlated relaxation rates across the glycosidic bond in DNA nucleotides [2] and corrections of the NMR spin-spin couplings with respect to rotation in silylated phenols [3].

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**P27**

## NUCLEOTIDE BINDING INDUCES CONFORMATIONAL CHANGES APPARENT ON THE $\text{Na}^+/\text{K}^+$ -ATPASE LARGE CYTOPLASMIC LOOP

L. Šimunová<sup>1,3</sup>, Z. Lánský<sup>1,2</sup>, M. Kubala<sup>5</sup>, J. Teisinger<sup>1</sup> and E. Amler<sup>3,4</sup>

<sup>1</sup>*Institute of Physiology, Academy of Sciences of Czech Republic*

<sup>2</sup>*Institute of Physics, Charles University, Czech Republic*

<sup>3</sup>*Institute of Biophysics of the 2<sup>nd</sup> Medical School, Charles university, Czech Republic*

<sup>4</sup>*Institute of Experimental Medicine, Academy of Sciences of Czech Republic*

<sup>5</sup>*Department of experimental physics, Faculty of Science, Palacky University, Czech Republic*

The ATP binding site of the  $\text{Na}^+/\text{K}^+$ -ATPase is located on the large cytoplasmic loop ( $\text{H}_4$ - $\text{H}_5$  loop) of the alpha-subunit. Conformational changes of the  $\text{H}_4$ - $\text{H}_5$  loop has been observed upon the ATP binding as well as the  $\text{Na}^+/\text{K}^+$ -ATPase association in the presence of  $\text{MgATP}$ . In this study we have analyzed the changes in conformation and quaternary structure of the  $\text{H}_4$ - $\text{H}_5$  loop by the means of site-directed mutagenesis and fluorescence spectroscopy. DNA sequence of a large cytoplasmic loop of  $\text{Na}^+/\text{K}^+$ -ATPase alpha-subunit was subcloned into expression vector pET28b and expressed as a  $(\text{His})_6$ -tag fusion protein in *E. coli* BL21 cells. We have prepared a set of single-tryptophan mutants mapping the surface of the  $\text{H}_4$ - $\text{H}_5$  loop.  $\text{Mg}$ ,  $\text{MgATP}$ ,  $\text{NaATP}$  titration experiments were performed with these point mutants and the fluorescence emission was used to monitor changes of polarity in the tryptophan microenvironment caused by the substrate

binding, thus monitoring the changes of the protein conformation. Time resolved fluorescence measurements were performed with selected point-mutants to specify more closely the effects of the ligand binding on the protein conformation and changes in quaternary structure. Results of the fluorescence experiments were thereafter interpreted in terms of previously released computer model of the  $\text{H}_4$ - $\text{H}_5$  loop [1].

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## MOLECULAR BASIS OF A LIPID-PSBH PROTEIN INTERACTION: A STRUCTURAL AND "AB-INITIO" COMPUTATIONAL STUDY

Dalibor Štys<sup>1,2</sup>, Žofie Sovová<sup>1</sup>, Zbyněk Halbhuber<sup>1</sup>, and Rüdiger Ettlich<sup>1</sup>

<sup>1</sup>Laboratories of High Performance Computing and Biomembranes, Institute of Physical Biology of the University of South Bohemia, and Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, Zámek 136, 373 33 Nové Hradky, Czech Republic

<sup>2</sup>Department of Autotrophic Microorganisms, Institute of Microbiology CAS, 37901 Trebon - Opatovický mlyn, Czech Republic, Email: stys@jcu.cz

Interaction of the PsbH protein of photosystem II was analysed by combination of CD and NMR spectroscopy and computer modeling. CD and NMR spectroscopy indicated that interactions to of PsbH to all bilayer-forming lipids, including those extracted directly from cyanobacterial thylakoid membranes, interact with PsbH non-specifically. The molecular dynamic calculation of PsbH-bilayer form-

ing lipid interactions indicates that both salt-bridge formation and hydrogen bond formation in the lipid-water interface are feasible and stabilize fraction of at least 10 lipid molecules in the neighbourhood of the protein. This may lead to formation of lipid domains which were hypothesized to be involved in assembly of membrane protein complexes.

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## PROTEIN-PROTEIN INTERACTIONS DURING EARLY SPORULATION EVENTS IN BACILLUS SUBTILIS

K. Šurdová, Z. Chromíková, I. Barák

*Institute of Molecular Biology, Slovak Academy of Sciences, 845 51 Bratislava*

Sporulation is an excellent example of prokaryotic differentiation. During the sporulation, *B. subtilis* goes through asymmetric division. Thus, bacterium gives rise to two diverse cells with different fates: the smaller prespore which develops into highly resistant spore, and the larger 'mother' cell which is predetermined to abolish. Different gene expression profiles are provided by sequential activation of compartment-specific sigma factors.

$\sigma^F$  is the first compartment-specific sigma factor. However, it is expressed well before the asymmetric septation, but becomes active only in the prespore [1]. Being the first one in a cascade of compartment-specific factors, the activation of  $\sigma^F$  is crucial for normal progression in sporulation. Proteins participating in the spatial and temporal control of  $\sigma^F$  activity are also expressed in the predivisional sporangium and include SpoIIE, SpoIIAA and SpoIIAB. SpoIIAB is an anti-sigma factor that interacts with  $\sigma^F$  and retains it in an inactive form [2]. This inhibition is perturbed by SpoIIAA protein. When phosphorylated, SpoIIAA is not able to interact with SpoIIAB. To release  $\sigma^F$  from an inhibitory complex, SpoIIE phosphatase dephosphorylates SpoIIAA~P which, in turn, activates  $\sigma^F$  [3,4].

SpoIIE is a transmembrane protein with dual function. Its phosphatase activity accounts for  $\sigma^F$  activation, while the central domain of SpoIIE is involved in asymmetric septum formation [5]. This is accomplished via the interaction with its binding partner FtsZ, which is the most important underlying protein for cell division and forms tubulin-like protofilaments. SpoIIE switches on a cascade of reactions that permit cell to continue in sporulation. This

activity is restricted purely to the prespore compartment by its phosphatase function. Being a key regulatory protein, the question is, how the action of SpoIIE is regulated.

Contradictory results account for not unified theory of  $\sigma^F$  activation and the mechanism of its timing and spatial control. Many biochemical studies have been executed, but there still remain uncertainties.

For elucidation, two approaches are to be employed to study protein-protein interactions: AFM and SPR. Both procedures are preceded by the isolation of proteins, using heterologous *E. coli* expression systems and a variety of purification steps. Thus, purification of proteins was our first principal goal. We also report the improvements in SpoIIE isolation and purification steps that emerge to be beneficial not only for biochemical but also for crystallographic studies.

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P30

## STRUCTURAL STUDY ON CRYSTAL OF HEME BINDING PROTEIN FROM PHOTOSYNTHETIC ANAEROBIC BACTERIUM *THIOCAPSA ROSEOPERSICINA*

I. Tomčová<sup>1,2</sup>, R. M. M. Branca<sup>3</sup>, Cs. Bagyinka<sup>3</sup>, G. Bodo<sup>3</sup> and I. Kutá Smatanová<sup>1,2</sup>

<sup>1</sup>*Institute of Physical Biology, University of South Bohemia at České Budějovice, Zámek 136, CZ-373 33 Nové Hradky, Czech Republic*

<sup>2</sup>*Institute of Systems Biology and Ecology, Academy of Sciences of the Czech Republic, Zámek 136, CZ-373 33 Nové Hradky, Czech Republic*

<sup>3</sup>*Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62, HU-6726 Szeged, Hungary  
e-mail: tomcova@ufb.jcu.cz*

**Keywords:** cytochrome, electron transfer, crystallization, diffraction

### Introduction

The cytochromes are ubiquitous proteins present in all living organisms and involved in a variety of intracellular processes that are essential for life. Most notable is their participation in electron transfer reactions, usually as components of a complex reaction pathway, necessary for the production of energy either through oxidation of metabolites or via photosynthesis. Cytochromes are members of a larger class of proteins, known as hemoproteins. The hemoproteins derive their name from the presence of one or more iron porphyrin prosthetic groups (called as hemes). Besides cellular bioenergetics, the heme is also involved in ligand binding reactions necessary for oxygen transport [1].

Compared with other biologically active molecules, cytochromes are some of the simplest bioinorganic compounds considering of molecular weight and structure. The active center of cytochromes is the heme group [2]. It consists of a porphyrin ring chelated to an iron atom. The porphyrin ring is a macrocyclic pyrrole system with conjugated double bonds. These compounds undergo chemical oxidation and reduction, cycling between ferrous ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) forms, in contrast to the hemoglobin, where the iron is normally in the ferrous ( $\text{Fe}^{2+}$ ) state.

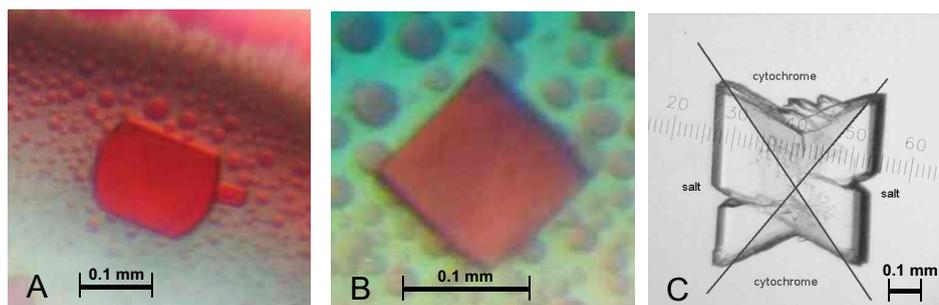
Our cytochrome is characterised by alpha-peak wavelength of 553 and a molecular mass of 25 kDa. The absorption spectrum (performed by UV-visible absorption spectroscopy [3]) presents distinct split alpha band, and a very low alpha to beta ratio. The protein consist of two heme molecules in single polypeptide chain with classical

Cys–X–Y–Cys–His heme binding sites. The fifth heme iron ligand is always provided by a histidine residue [2]. Cytochrome is located probably in bacterial periplasmic space of *Thiocapsa roseopersicina* cell wall. The established properties of this hemoprotein indicate that it belongs to the  $c_4$  family [3] of diheme cytochromes. It is the first cytochrome of this class that comes from an anaerobic organism. Due to its important function, it is of essential interest to study structural features of cytochromes using X-ray crystallography.

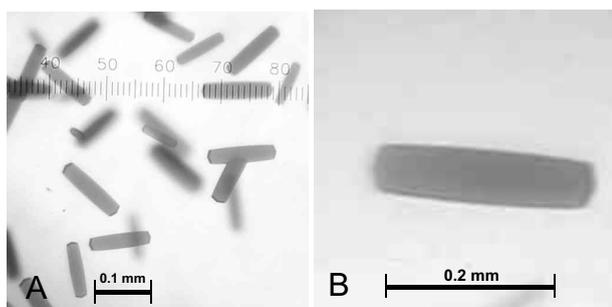
### Materials and crystallization methods

Cytochrome  $c_4$  (cyt  $c_4$ ) from the purple photosynthetic bacterium *Thiocapsa roseopersicina* was isolated and purified according to [3]. The bacterium has four different hydrogenases and three different cytochromes. The cyt  $c_4$  has been studying by crystallographic, proteolytic [4] and spectroscopic methods. Cyt  $c_4$  was crystallized using standard crystallization methods based on vapor diffusion (hanging and sitting drops [5]) and advanced crystallization method based on the counter-diffusion (crystallization in capillaries [6]). Initial crystallization trials with ammonium sulfate (AS) yielded pseudocrystals as red thin plate (Figure 1) with components from 0.1 M sodium chloride and 0.1 M citric acid pH 6.0 in the reservoir solution. Ranging pH value higher than 7.5 the phase separation of protein appeared.

Crystallization trials were performed at 20 °C. After fine-tuning crystallization conditions, the most suitable concentration of protein (10–15 mg/ml) and the percentage of precipitation agent were found. The first suitable crystal growth was observed at pH 6.0 (Figure 2) using the addition of metal ions –  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ba}^{2+}$  (from Hampton



**Figure 1:** A, B – Pseudocrystals of cyt  $c_4$  and C – crystal of cyt  $c_4$  constructed together with AS.



**Figure 2:** A, B – Crystals of cyt  $c_4$

Research Additive Screen HR2–428). Cyt  $c_4$  crystals were grown in capillaries when the precipitating system contacts the protein solution because a wave of supersaturation was triggered.

#### Diffraction measurement

The monocrystals of cyt  $c_4$  were tested at the home source diffractometer at LEC (University of Granada) and measured at synchrotron DESY (Hamburg) in the loops and in the capillaries directly. Structure of cyt  $c_4$  will be solved using molecular replacement method.

Colored crossbred plates of holoprotein crystals with dimensions of approximately 200 50 30  $\mu\text{m}$  grew within 3–4 days in several conditions. Protein crystals grown in capillaries were measured directly at synchrotron DESY (Hamburg), beamline X11. Crystallization conditions are now optimized in order to prepare monocrystals of cyt  $c_4$  suitable for X-ray structural analysis.

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## STRUCTURAL NON-RIGIDITY OF HETERO-AROMATIC BASES IN NUCLEIC ACIDS

L. Trantírek<sup>1</sup>, S. Trantírková<sup>1</sup>, B. Schneider<sup>2</sup>, V. Sychrovský<sup>2</sup>, N. Špačková<sup>3</sup>, J. Šponer<sup>3</sup>

<sup>1</sup>*Institute of Biological Sciences, University of South Bohemia and Institute of Parasitology AS CR, Branisovska 31, Ceske Budejovice, Czech Republic*

<sup>2</sup>*Institute of Organic Chemistry and Biochemistry AS CR, Flemingovo namesti 2, Prague, Czech Republic*

<sup>3</sup>*Institute of Biophysics AS CR, Kralovopolska 135, Brno, Czech Republic, E-mail: trant@bf.jcu.cz*

Deviations from ideal  $sp^3$  and  $sp^2$  geometry at C1' and N1/9 sites were analyzed for about 400 nucleosides extracted from crystallographic structures of nucleic acids derived at ultra-high (atomic) resolution. The influence of the structural non-rigidity of nucleic acid bases on parameterizations of Karplus-like relationships for NMR

scalar coupling constants and CSA/DD cross-correlated relaxation rates across the glycosidic bond will be discussed.

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**IN-CELL NMR IN *Leishmania tarentolae*****S. Trantírková<sup>1</sup>, V. Dotsch<sup>2</sup>, K. Alexandrov<sup>3</sup>, R. Breitling<sup>3</sup>, L. Trantírek<sup>1</sup>**<sup>1</sup>*University of South Bohemia, Faculty of Biological Sciences, Ceske Budejovice (Czech Republic)*<sup>2</sup>*W. Goethe University, Institute for Biophysical Chemistry and Center for Biomolecular Magnetic Resonance, Frankfurt (Germany)*<sup>3</sup>*Planck Institute for Molecular Physiology, Department of Physical Biochemistry, Dortmund (Germany),  
E-mail: trant@bf.jcu.cz*

To assess the influence of the native-like environment and a drug binding on a protein tertiary structure, we are developing a methodology for acquiring *in vivo* high-resolution NMR spectra of the proteins over-expressed inside *L. tarentolae* cells. *L. tarentolae*, in contrast to other expression systems such as *E. coli* or baculovirus, possess mammalian-type of post-translational modifications and full protein folding machinery. The parameters critical for

observing proteins inside living *L. tarentolae* cells using NMR spectroscopy are evaluated.

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**PURIFICATION AND PREPARATION OF ANTIBODY SCFV TU20 P 643 AND P 745 FOR CRYSTALLIZATION AND DETERMINATION STRUCTURE BY X-RAY****B. Uhnáková\*, J. Brynda, M. Fábry, M. Hořejší, V. Král, I. Siegllová, J. Sedláček***Department of Recombinant Expression and Structural Biology, Institute of Molecular Genetics, Flemingovo nám. 2, CZ-166 37 Praha 6*

The class III beta-tubulin isotype is widely used as a neuronal marker in normal and neoplastic tissues [1]. This isotype was, however, also immunodetected in certain tumours of non-neuronal origin such as squamous cell carcinoma. The distribution of class III beta-tubulin in normal and neoplastic tissues was compared using a newly described monoclonal antibody. The TU-20 mouse monoclonal antibody was prepared against a conserved synthetic peptide from the C-terminus of the human class III beta-tubulin isotype. Its specificity was confirmed by immunoblotting and by immunofluorescence microscopy on cultured cells. The results indicate a specific TU-20 epitope expression exclusively in neuronal tissues. The antibody could thus be a useful tool for the probing of class III

beta-tubulin functions in neurons as well as immunohistochemical characterisation.

Fv fragments are the smallest antibody molecules that still retain the entire antigen-binding site. In single chain Fv fragments (scFv), variable domains are joined by a flexible linker. We have prepared scFv TU20 construct and screened its activity (ELISA). Our objective has been to study the activity and continue to structural studies with improvement in the protein engineering. Two constructs, scFv TU20 p643 and p745 (contains Tyrosine rich domain) have been cultivated and purified for crystallization.

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## STRUCTURAL STUDIES OF LIGAND BINDING OF NATURAL KILLER CELL RECEPTOR, PROTEIN CD69

O. Vaněk<sup>1</sup>, P. Novák<sup>2</sup>, V. Kopecký Jr.<sup>3</sup>, V. Křen<sup>2</sup>, J. Brynda<sup>4</sup>, K. Bezouška<sup>1,2</sup>

<sup>1</sup>Dept. of Biochemistry, Faculty of Science, Charles University in Prague, Hlavova 8, CZ-12840 Prague 2

<sup>2</sup>Institute of Microbiology, Academy of Sciences of the Czech Republic, Videnska 1083, CZ-14220 Prague 2

<sup>3</sup>Institute of Physics, Faculty of Mathematics and Physics, Charles University in Prague, Ke Karlovu 5, CZ-12116 Prague 2

<sup>4</sup>Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Flemingovo namesti 2, CZ-16636 Prague 6

CD69 protein [1], an early activation antigen of human lymphocytes, is one of the most studied surface receptor molecules involved in tumour recognition by natural killer cells. Although there is certain knowledge about its function from immunological point of view, detailed structural description of target recognition by natural killer cells is missing, including identification of CD69 natural ligand. CD69 receptor belongs to a group of C-type lectin-like lymphocyte receptors and till now several types of possible ligands have been identified or proposed: calcium cation, various carbohydrate structures and even peptides. Crystal structures of carbohydrate recognition domain (CRD) of CD69 molecule have been already published [2,3], but crystallization conditions used does not favour ligand binding. In this work we present our structural approach to describe CD69 binding properties. After recombinant production and optimization of *in-vitro* refolding of soluble CRD domain of CD69 protein, we analyzed its homogeneity by FT-ICR mass spectrometry and its secondary structure was determined by drop coating deposition Raman spectroscopy method [4]. Further, ligand binding was examined both by Raman spectroscopy and by protein crystallization. We tested several types of proposed CD69 ligands, from single monosaccharide ligand *N*-acetyl-D-glucosamine, to more complex structures, such as antennary oligosaccharides isolated from hen egg white pro-

tein ovomucoid, synthetic peptidomimetic ligands based on calixarene core, or heptapeptide ligand from mycobacterial heat shock protein hsp60, which all binds with high affinity to CRD domain of CD69 receptor. Comparison between computational docking model of calcium binding and observed results by crystallography is discussed.

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## COMBINATION OF X-RAY DIFFRACTION ANALYSIS AND LIMITED PROTEOLYSIS USED IN STRUCTURAL STUDY OF FLAVOPROTEIN WrbA

J. Wolfová<sup>1,2</sup>, J. Carey<sup>3</sup> and I. Kutá Smatanová<sup>1,2</sup>

<sup>1</sup>*Institute of Physical Biology, University of South Bohemia České Budějovice, Zámek 136, CZ-373 33 Nové Hradý, Czech Republic*

<sup>2</sup>*Institute of Systems Biology and Ecology, Academy of Science of the Czech Republic, Zámek 136, CZ-373 33 Nové Hradý, Czech Republic*

<sup>3</sup>*Chemistry Department, Princeton University, Washington Rd and William St, Princeton, NJ 08544-1009, USA*

The tryptophan (W)-repressor binding protein A (WrbA) identified as an *Escherichia coli* stationary-phase protein belongs to a new family of multimeric flavodoxin-like proteins implicated in oxidative-stress defense. Based on the computational studies and biochemical experiments it was shown that the WrbA protein shares the open, twisted / fold with flavodoxins and its physiological cofactor is the flavin mononucleotide (FMN) as well [1, 2]. Since WrbA is largely uncharacterized with respect to both molecular and physiological functions and displays some interesting structural properties representing the new family, the present effort is aimed at structural characterization by X-ray diffraction analysis and limited proteolysis.

The WrbA apoprotein was successfully crystallized using standard vapor diffusion methods and counter-diffusion methods in single capillaries [3]. Optimization of crystallization conditions by using additives, especially Cd<sup>2+</sup> and Li-citrate, led to getting single crystals suitable for diffraction measurements [4]. The crystals diffracted to a resolution of 2.2 Å at synchrotrons DESY (X13) in Hamburg (Germany), and Elletra (XRD1) in Trieste (Italy), but the diffraction data were not sufficiently good for solving structure. Additional optimization of crystallization conditions of the WrbA apoprotein is in progress. The WrbA protein in complex with the cofactor, FMN, has also been crystallized, but the crystallization has to be optimized yet.

For a complementary investigation of the folding architecture of the WrbA protein the proteolytic dissection method, called limited proteolysis, was applied. It is an important method used for identification and characterization of the folded substructures of proteins and their stability [5]. Although the WrbA protein contains many potential cleavage sites for the proteases used, the intact protein was attacked by proteases only at several specific sites, which resulted in several protein fragments identified by the SDS-PAGE. The presence of the folded structure in these fragments was confirmed by CD spectroscopy. This result is in agreement with the theoretical assumption that only

the accessible cleavage sites lying in the incompletely ordered parts of protein structure are attacked by proteases while the cleavage sites buried inside the folded structure remain intact. By the limited proteolysis a fragment of nearly the same size as the intact protein was generated. This result indicates that the WrbA protein contains a flexible part at one end. Removing of this part of protein might improve crystallizability of the protein. The experiments showed also that the FMN binding increases the protein stability, which was indicated by the longer resistance of the intact protein to protease activity. Sequencing of the fragments produced by proteolytic digestion is under way, promising to shed light on the folding of WrbA protein and to identify the part of protein causing the crystallization difficulties. The results reported here show that the limited proteolysis could serve as a competent accessory to X-ray diffraction study.

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