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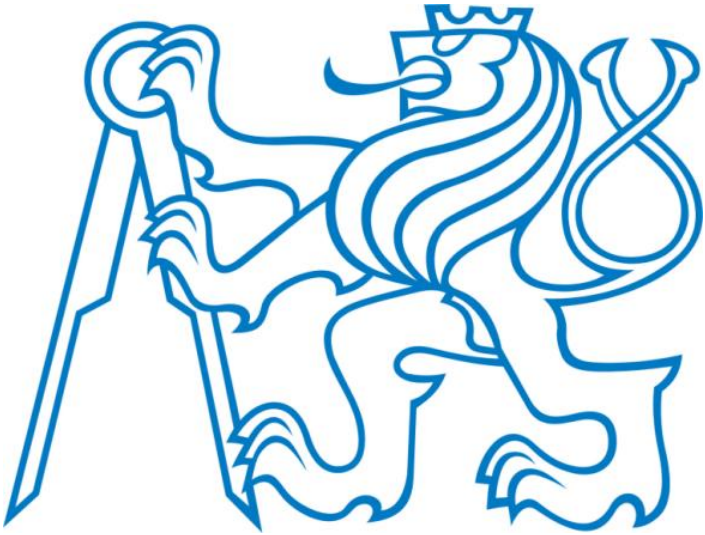

Doctoral thesis summary

CZECH TECHNICAL UNIVERSITY IN PRAGUE

FACULTY OF NUCLEAR SCIENCES
AND PHYSICAL ENGINEERING

DEPARTMENT OF SOLID STATE PHYSICS

Ing. et Ing. Andrea Štěpánková

X-ray structural analysis of enzymes from extremophiles and their complexes with bound ligands

Doctoral study program: Physical engineering – Solid state engineering

Abstract of the doctoral thesis submitted for the academic degree "Doctor", in abbreviation "Ph.D."

Prague, February 2012

ČESKÉ VYSOKÉ UČENÍ TECHNICKÉ V PRAZE

FAKULTA JADERNÁ A FYZIKÁLNĚ INŽENÝRSKÁ

KATEDRA INŽENÝRSTVÍ PEVNÝCH LÁTEK

Ing. et Ing. Andrea Štěpánková

Rentgenová strukturní analýza enzymů z extremofilů a jejich komplexů s ligandy

Doktorský studijní program: Aplikace přírodních věd

Studijní obor: Fyzikální inženýrství - Inženýrství pevných látek

Teze k disertační práci k získání akademického titulu "doktor", ve zkratce "Ph.D."

Prague, February 2012

Disertační práce byla vypracována v prezenční formě doktorského studia na Katedře inženýrství pevných látek Fakulty jaderné a fyzikálně inženýrské ČVUT v Praze.

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Teze byly rozeslány dne: .....

Obhajoba disertace se koná dne ..... v …… hod. před komisí pro obhajobu disertační práce ve studijním oboru **Fyzikální inženýrství - Inženýrství pevných látek** v zasedací místnosti č ..... Fakulty jaderné a fyzikálně inženýrské ČVUT v Praze.

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Anotation: The submitted thesis is focused on determination of the structures of two types of enzymes from extremophiles. Extremophiles are organisms inhabiting extreme conditions on Earth. Psychrotrophs possess the minimal temperature of growth around 0 °C and the optimal one around 20 °C. Halophiles require at least 0.2 M concentrations of salt (NaCl) for their growth. As one of two results of the thesis, three atomic structures of complexes of β-galactosidase enzyme from psychrothropic bacterium *Arthrobacter* sp. C2-2 with three various ligands have been determined. The β-galactosidase enzyme catalyzes hydrolysis of the galactosyl moiety from non-reducing termini of oligosaccharides. These complexes proved the activity of the enzyme in the crystal. As the other result of the thesis, three atomic structures of organophosphorus acid anhydrolase enzyme from slightly halophilic bacterium *Alteromonas macleodii* have been determined. Moreover, mutagenesis and enzyme assays of this enzyme from psychrothropic and slightly halophilic bacterium *Pseudoalteromonas haloplanktis* have been carried out. This enzyme catalyzes hydrolysis of several types of highly toxic organophosphate compounds which are commonly used as pesticides or as nerve agents, namely: fluoride-containing sarin and soman and cyanide-containing tabun. The thesis is first what presents the whole atomic structure of this enzyme.

Anotace: Předmětem odevzdané disertační práce jsou strukturní studie dvou typů enzymů z extremofilních organismů, což jsou organismy tolerující nebo potřebující pro svůj život extrémní podmínky. Psychrotropní organismy mají minimální teplotu růstu přibližně 0 °C a optimální přibližně 20 °C. Halofilní organismy vyžadují alespoň 0,2 M koncentraci soli (NaCl) ke svému růstu. Jedním z výsledků této disertační práce jsou tři struktury komplexů enzymu β-galaktosidasa z psychrotropní bakterie *Arthrobacter* sp. C2-2 s navázanými ligandy. Enzym β-galaktosidasa katalyzuje hydrolýzu galaktosilních jednotek z oligosacharidů. Tyto komplexy potvrzují aktivitu enzymu v krystalu. Dalším výsledkem této disertační práce jsou tři struktury enzymu organofosfátové anhydrolasy ze slabě halofilní bakterie *Alteromonas macleodii*. Navíc jsou prezentovány výsledky mutageneze a měření enzymové aktivity tohoto enzymu z psyhrotropní a slabě halofilní bakterie *Pseudoalteromonas haloplanktis*. Tento enzym katalyzuje hydrolýzu několika typů vysoce toxických organofosfátových sloučenin, které jsou široce používány jako pesticidy nebo jako bojové plyny, jmenovitě: sarin, soman nebo tabun. Význam disertační práce je především v tom, že je prvně určena celá atomová struktura tohoto enzymu.

1. Current state of research field

Proteins are important biological macromolecules present in all organisms and have a specific function. Proteins are polymers of residues of 20 different amino acids. Determination of atomic structure of proteins and their complexes helps to understand the mechanism of particular processes they are involved in.

X-ray protein crystallography is a powerful tool for studying the structure and function of proteins. Investigation of the atomic structure of a complex of an enzyme and a small molecule can improve our knowledge of possible interactions and reaction mechanisms which such enzyme catalyzes.

The major limiting factor of X-ray protein crystallography consists in preparation of good quality macromolecular crystals suitable for diffraction analysis. Protein crystallography is a multidisciplinary field involving these elementary steps: protein expression and purification, characterization of the prepared protein solution, growing crystals from the prepared and purified protein solution, diffraction data collection and processing them, solving the phase problem and refining the whole protein structure.

The thesis is focused on determination of the structures of two types of enzymes from **extremophiles**. Extremophiles are organisms inhabiting extreme conditions on Earth (for illustration: hydrostatic pressures of 120 MPa, temperatures of -50 °C or 113 °C or pH values of 0.5 or 12.0).

Psychrotrophs are a group of extremophilic microorganisms with the minimal temperature of growth around 0 °C and with the optimal temperature of growth around 20 °C. [1]

Halophiles are a group of extremophilic microorganisms requiring at least 0.2 M concentrations of salt (NaCl) for their growth. [2]

Many enzymes from extremophiles are interesting for their ability to catalyze reactions under extreme conditions contrary to their mesophilic counterparts. Because of their activity, they are expected to be efficient catalysts for industrial purposes and to be a link between biological and chemical process.

***Arthrobacter* sp. C2-2** is a psychrotrophic gram-positive soil bacterium from genus *Arthrobacter*, adapted to growth at low temperatures isolated in the Antarctic area and its cardinal growth temperatures were determined as: minimal is lower than 2 °C, optimal is a range between 20 – 22 °C and the maximal is 33 °C. [3]

Alteromonas macleodii is a slightly halophilic marine bacterium from genus *Alteromonas* and can grow optimally in salt concentrations of 1.5 to 3.5%

NaCl [4]. It is one of the most common heterotrophic bacteria living in open marine water and cells are gram-negative, non-spore forming, straight rods with 0.7 – 1 μm diameter, 2 – 3 μm long, motile by means of a single unsheathed polar flagellum, not luminescent, not pigmented, strictly aerobic, chemoorganotroph, oxidase positive and catalase negative.

Pseudomoalteromonas haloplanktis is a fast growing psychrotrophic and slightly halophilic marine bacterium from genus *Pseudoalteromonas*, adapted to growth at low temperatures and in salt concentrations of 1.5 to 3.5% NaCl. [5], [6] The cells are gram-negative, non-spore-forming, motile by means of single unsheathed polar flagella, not luminescent, strictly aerobic, oxidase positive but catalase activity is generally weak and irregular.

β-Galactosidase (**β-Gal**) (**β-D-galactoside galactohydrolases**, EC 3.2.1.23) catalyses hydrolysis of the galactosyl moiety from non-reducing termini of disaccharide substrates into monosaccharides. β-Galactosidase activity is an essential in the human body, its deficiencies may result in digestive insufficiency (lactose intolerance) [7] or illnesses as galactosialidosis [8] or Morquio B syndrome [9]. The β-gal enzyme has two main biotechnological applications in the dairy industry — production of galactooligosaccharides for use in probiotic foodstuffs [7] and preparation lactose-free milk for lactose-intolerant persons [7].

Organophosphorus acid anhydrolase (**OPAA**) (**diisopropyl-fluorophosphate fluorohydrolase**, EC 3.1.8.2) catalyses hydrolysis both dipeptides with proline residue at C-terminus and a wide variety of toxic organic compounds containing phosphorus and causing neuromuscular paralysis [10]. The toxic organophosphate compounds are widely used as pesticides in agriculture and as chemical warfare nerve agents. Enzymatic detoxification is more effective, economical and safe then other ways (e.g. incineration). The OPAA enzyme is considered for the purpose of detoxification of the highly toxic OPs [10], [11].

2. Goals and purpose of this thesis

This work is a part of the long-term project focusing on determination of three-dimensional structures of two biotechnologically-applicable enzymes active under extreme conditions and their complexes with ligands. The thesis consists of two main topics related to two types of enzymes:

A) Enzyme **organophosphate acid anhydrolase** from marine **slightly halophilic** bacterium *Alteromonas macleodii* and from marine **slightly halophilic** and **psychrothropic** bacterium *Pseudoalteromonas haloplanktis* is bifunctional and is able to hydrolyse both dipeptides Xaa-Pro and toxic organophosphates. **One of two aims of the thesis is to determine the whole structure of this enzyme by methods of protein crystallography.**

B) Enzyme **β-galactosidase** from **psychrothropic** bacterium *Arthrobacter* sp. C2-2 is cold-adapted and thus is able to hydrolyse lactose under lower temperature which is a desirable property for dairy industry. **The other aim of the thesis is to describe interactions between ligands and residues in its active site by method X-ray structural analysis.**

In order to reach both of the above described aims following sub-aims were prescribed:

Enzyme organophosphate acid anhydrase from marine bacteria

- *Alteromonas macleodii*
- *Pseudoalteromonas haloplanktis*

1. Enzyme characterisation (DLS, gel-electrophoresis, size exclusion chromatography)
2. Enzyme crystallisation and optimization of crystallization conditions
3. X-ray data collection and processing, solving the phase problem and the structure determination
4. Analysis of the structure

Enzyme β-galactosidase from *Arthrobacter* sp. C2-2

1. X-ray data processing and three structures of the enzyme with bound ligands determination and refinement
2. Analysis of the structures of the complexes

3. Materials and Methods and Results

1. Cloning, expression and purification

Protein samples of **phOPAA** (organophosphorus acid anhydrolase from *Pseudoalteromonas haloplanktis*) and **amOPAA** (organophosphorus acid anhydrolase from *Alteromonas macleodii*) wild type were obtained from Novozymes a./s. company and were prepared as described in the patent WO 2009130285-A1 [12].

Protein samples of **aC22-β-gal** (β-galactosidase from *Arthrobacter* sp. C2-2) were obtained from Institute of Chemical Technology Prague and were prepared as described previously [13].

2. Site-directed mutagenesis

As a result of the site-directed mutagenesis seven variants of the **phOPAA** enzyme were prepared**: Y212F, Y212S, H226N, H226K, H334N, H334K and H334Q**.

The *phopaa* plasmid was modified according to [14] to prepare variants of the phOPAA enzyme. The genes were amplified using the PCR methods under the same conditions as for the phOPAA wild type [12]. Mutations in the *phopaa* gene were generated using the QuickChange site-directed mutagenesis kit [15] and the mutagenic primers. The achievements of the site-directed mutagenesis procedure were confirmed by determining the nucleotide sequences of the whole genes. The phOPAA mutants were overexpressed in *E. coli* strain BL21 containing derivative plasmids [12]. Variants were concentrated and stored in 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.5) at 5 °C.

3. Enzyme assay

The prolidase activity of the **phOPAA wild type** and **mutants** was measured using a series of Xaa-Pro dipeptides with Pro on the C-terminus (Ala-Pro, Leu-Pro, Phe-Pro) as described in [16]. The changes in absorbance at 222 nm as a consequence of the peptide bond hydrolysis were monitored. Eight different Britton-Robinson buffers (pH interval 3-10) [17] were used for determining pH optimum of the phOPAA wild type and mutants. The most

suitable dipeptide substrate and the pH optimum were set by comparing the obtained reaction rates.

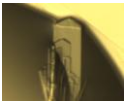
According to the results the best substrate for the **phOPAA wild type** is Ala-Pro. In the case of substrates Ala-Pro or Leu-Pro the pH optimum is pH 8, for Phe-Pro as substrate is shifted a little bit from basic pH to neutral pH on level pH 7.

The **mutants Y212S, H226K, H334N, H334K** and **H334Q** were inactive. Activity was observed for **mutants Y212F** and **H226N** at higher value pH comparing to the wild type phOPAA. Their pH optimum is shifted slightly to more basic pH on level pH 9 compared to the wild type phOPAA.

4. Crystallization

The **amOPAA** enzyme was crystallized by the vapor diffusion method in hanging drops. The protein solution contained 50 mM NaH_2PO_4/Na_2HPO_4 (pH 7.5), 1 mM NaN_3 and 13.4 mg/ml protein. Suitable crystallization conditions are summarized in Table 1. Prior X-ray measurements, the crystals were protected by optimized cryo-solution composed of 25% (*v/v*) ethylene glycol and flash frozen in the liquid nitrogen.

Table 1: The found crystallization conditions producing diffracting crystals of the amOPAA enzyme. All crystals were grown at 18 °C in hanging drop by vapour-diffusion methods.

C1		0.056 M NaH_2PO_4 1.344 M K_2HPO_4 pH 8.2	*good diffraction*
C2		12 % *w/v* PEG 20000 0.1 M MES pH 6.5 0.1 M Ammonium sulfate	*good diffraction*
C3		17 % *w/v* PEG 10000 0.1 M Bis-Tris pH 5.5 0.1 M Ammonium acetate	*good diffraction*
C4		Polyacrylic acid sodium salt 5100 0.2 M $MgCl_2 . 6H_2O$ 0.1 M HEPES pH 7.5	*good diffraction*

The **aC22-β-gal** enzyme was crystallized by the vapor diffusion method in hanging drops previously described in [13]. Three wedge-like three-dimensional crystals were soaked in three various ligands: D-galactonolactone, β-D-galactose and isopropyl-β-D-thiogalactopyranoside (IPTG). The soaking time was 120 s for each experiment. Before flash freezing all crystals were soaked in optimized cryo-solution composed of 30% (*v/v*) ethylene glycol and 10 mM solution of particular ligand.

5. X-ray data collection, processing and structures determination

The structure solution process of **amOPAA** started by attempts to solve the phase problem by experimental phasing. Two different MAD data sets were collected on two different crystals (C3 and C4 according to Table 1) using four various wavelengths – native (1.91814 Å), peak (1.893 Å), inflection (1.894 Å), high remoted (1.850 Å). Manganese ion was considered as an element for anomalous scattering. The presence of Mn anomalous signal in the MAD data sets were confirmed but it was not sufficient for successful structure determination. In the course of further phasing efforts the structure of aOPAA-JD6.5 [18] became available. The structure of amOPAA was determined by the molecular replacement method with aOPAA-JD6.5 (PDB ID: 3L24 [18]) as a model.

Table 2: Overview of the determined and presented structures of amOPAA:

structure name	Resolution [Å]	crystallization condition	No. of monomer in AU	ligand
amOPAA-1	1.8	0.056 M Sodium phosphate monobasic monohydrate 1.344 M Potassium phosphate dibasic	1	—
amOPAA-Pro-Gly	1.9		1	Pro-Gly
amOPAA-3	2.1	0.1 M AS, 0.1 M MES pH 6.5, 12 % *w/v* PEG 20 000	2	—

Three various structures were determined (Table 2). The structures were solved up to resolutions: 1.8 Å, 1.9 Å and 2.1 Å. The structures were refined to the final *R*-factor and R_{free} factor 15.6 % and 18.5% (amOPAA-1), 15.6 % and 20.1% (amOPAA-Pro-Gly) and 18.8 % and 23.9% (amOPAA-3). The asymmetric unit contains one monomer in the case of amOPAA-1 and amOPAA-Pro-Gly and one dimer in the case of amOPAA-3. The functional enzyme is composed of two chains.

The amOPAA monomer is composed of two domains (Fig. 1a). The smaller N-terminal domain is composed of residues 1 – 160 and is also called the N-domain and is composed of five α-helixes and five β-strands. The larger C-terminal domain comprises residues 161 – 445 is composed of 7 α-helixes and five β strands. Arrangement of the domains resembles the letter V, with the chain "bent" between the two domains. In contrast to the N-domain β-sheet, all the β-strands in the C-domain are antiparallel and form the "core" of the C-domain surrounded by the α-helical scaffold. The β-strands are long and strongly curved which evokes pita bread (firstly described in eMetAP [19]). The four longest α-helices run parallel to the β-strands and most of the other shorter helices form a cluster.

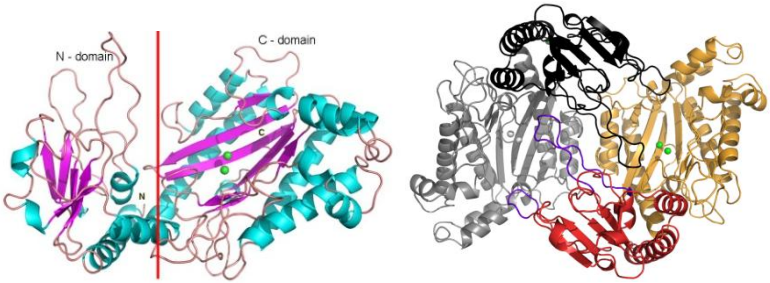


Figure 1: a) Structure of the monomer of organophosphorus acid anhydrolase from *Alteromonas macleodii*. The monomer colored according secondary structure: α helices – cyan, β strands – magenta, Mn^{2+} ions – light-green. b) The dimer of amOPAA consists of two chains denoted as A (colored chain) and B (grey chain). The N–domains are highlighted by saturated colour and the C-domains are colored in light color. Two fingers responsible for holding dimer together are colored purple in monomer A. Manganese ions are shown as spheres in the active site.

After fitting all side chains into electron density maps, the unmodelled blobs greater than 5 σ in $2F_o$- F_c were checked as a possible ligand electron density map in the case of amOPAA-Pro-Gly structure. The molecule of ligand was tracked in the potentially suspicious electron density in the active site. The position of ligand was then manually slightly modified.

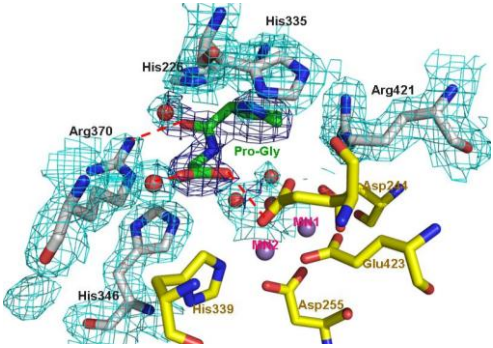


Figure 2: The view into the active site of the amOPAA-Pro-Gly structure with bound ligand.

Topology of monomer of **amOPAA** is the same in all three presented structures. The position of the first five residues differs: in the am-OPAA-1 and am-OPAA-Pro-Gly structures there is the nickel ion what cause a special arrangement of the first residues in contrast to am-OPAA-3, where is no evidence that nickel ion is present. Due to the main chain goes in different way and also positions of side chains differ. The positions of the rest 440 residues are almost the same, are well supported by electron density. All

residues were modeled in *trans* conformation. Two manganese ions were located in the each active site according to the strong $2F_o$-F_c maximum. Their mutual distances are almost the same and they are coordinated as the same way. Of other solvent molecules the structures contains localized water molecules and one phosphate anion per monomer. Only amOPAA-3 was determined as the dimer in the asymmetric unit.

The amOPAA coordinates and structure factors were deposited with the Protein Data Bank under accession code 3RVA.

The three-dimensional structures of **the complexes** formed between **enzyme aC22-β-gal** and three types of various ligands (**D-galactonolactone, β-D-galactose and IPTG**) have been solved and refined at 2.5 Å, 2.1 Å and 3.3 Å resolution. **Interactions of particular ligands with aC22-β-gal** have been analyzed and compared in all structures. These interactions have been also compared to interactions of these types of ligands with β-gal from *Escherichia coli* (ec-β-gal). The three determined complexes illustrate that there are two binding positions in the active site of aC22-β-gal. These binding positions are the same as described previously for ec-β-gal: "the shallow binding mode" and "the deep binding mode" [20].

Table 3: Overview of determined structures of aC22-β-gal*:*

Structure code	Resolution [Å]	Crystallization condition	No. of monomer in AU	Ligand
aC22-β-gal-lactone	2.2	20% (*w/v*) PEG 4000 200 mM NaCl, 200 mM $(NH_4)_2SO_4$ 100 mM sodium citrate (pH 5.6)	6	D-galactonolactone
aC22-β-gal-galactose	2.1		6	β-D-galactose
aC22-β-gal-IPTG	3.3		6	IPTG

The presented structures of the complexes aC22-β-gal have the same overall fold of the monomer and the hexamer. In spite of the significant differences in the resolution limits and quality of the $2F_o$-F_c it is possible to compare the side chains position of the residues in the active site in its surrounding. The molecules of D-galactonolactone and β-D-galactose are bound in the "deep binding" mode, very deep in the active site, near Trp568. The binding position is not exactly identical and the bound ligands evoked a various structural changes. The molecule of IPTG is bound in the "shallow binding" mode. IPTG is not so deep in the binding pocket compared to the two previous complexes and it is positioned directly over the side chain of Cys999 (at the entrance of the binding pocket).

The presence of the shallow binding position was not expected in aC22-β-gal contrary to the presumable presence of the deep binding position. According

to [21] the Trp999 residue in ec-β-gal at the entrance in the binding pocket is the key residue for binding in the shallow binding position. It is the key residue for binding, catalysis, and synthesis of allolactose and other substrates in β-gal. According to [21] the consequence of the substitution of this tryptophane by other types of residues is greatly decreasing of the affinity for glucose and also changing the activation thermodynamics and the rates of the catalytic reactions in ec-β-gal. The tryptophane at this position is conserved in many related β-galactosidases [21]. The tryptophane residue is also part of the binding sites of many other proteins binding sugars. By contrast to ec-β-gal, aC22-β-gal has the cysteine residue instead of the tryptophane at this position. Thus for a long time it was assumed that the shallow binding position in aC22-β-gal is absent.

Both D-galactonolactone and β-D-galactose all bind in the deep binding position. Both of them evoked structure changes in the surroundings of the active site compared to the unliganded enzyme. D-galactonolactone is bound in the 1-5 form. This form is very similar to β-D-galactose, the main difference is D-galactonolactone is planar at C1. This uncharged molecule has an electric dipole moment with its positive end near C1. Thus D-galactonolactone is the transition state analogue and its binding mimics the transition state binding. Although molecules D-galactonolactone and β-D-galactose are very similar the binding interactions are not identical and there are some subtle differences in the surroundings of the active site of the complexes: D-galactonolactone has the 2-substituent deeper by 0.7 Å and the other substituents by 0.2 – 0.5 Å compared to β-D-galactose.

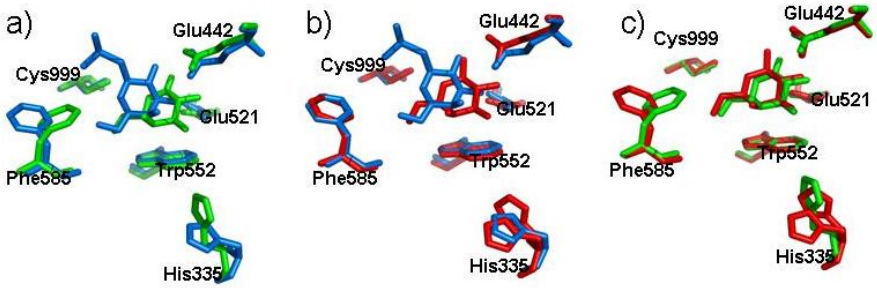


Figure 3: The comparison of the binding position of IPTG, D-galactonolactone and β-D-galactose in the active site of aC22-β-gal. The structure alignment of complexes with: a) IPTG and D-galactonolactone, b) IPTG and β-D-galactose and c) D-galactonolactone and β-D-galactose. The complex aC22-β-gal-IPTG is blue, aC22-β-gal-lactone is green and the aC22-β-gal-galactose is red. The conformation changes of Phe585 and His335 help stabilize the transition state and are only in aC22-β-gal-lactone.

4. Conclusion

The significant progress on the nerve agent biodegradation has been made during several last years. The **OPAA** enzyme is able to hydrolyze the highly toxic organophosphates (such as DFP, sarin, soman) [22] which are AChE inhibitors. These toxic OPs are commonly used as the **nerve agent** or as the **pesticides**. **The OPAA enzyme is considered for the purpose of detoxification of the highly toxic OPs** [18], [22].

This study is first one describing the whole structure of the "Mazur type" OPAA enzyme. This study presents three various structures of amOPAA [amOPAA-1 (monomer in the AU, Space group *C*2), amOPAA-Pro-Gly (monomer in the AU, space group *C*2, bound dipeptide ligand in the active site) and amOPAA-3 (dimer in the AU, space group $P2_12_12_1$)]. The presented structures amOPAA are very well determined and refined.

Moreover seven variants of the phOPAA enzyme were prepared: Y212F, Y212S, H226N, H226K, H334N, H334K and H334Q and their biochemical properties and the **enzyme activity against several selected dipeptides** were examined and compared to the phOPAA wild type results. The enzyme assay studies exposed loss of activity as a consequence of site-direct mutagenesis in Y212S, H226N, H334N, H334K and H334Q. Only the activity of Y212F and H226K sustained. Adding 100 mM NaCl and 10% (*v*/*v*) glycerol to the protein buffer helped to stabilize the mutant Y212F and its activity sustained several days. Moreover reactions catalyzed by Y212F with added NaCl or glycerol had higher rates comparing to reactions catalyzed by the mutant with no additives in the buffer solution. The pH optimum of the phOPAA wild type is between pH 7 and pH 8 depending on the dipeptide substrate type. The pH optimum of the Y212F and H226K mutants is shifted slightly to more basic pH on level pH 9 compared to the wild type phOPAA.

It is possible to influent the phOPAA activity and also to shift the pH profile by modification of the several amino acids. This study is able to clarify this behavior based on the known amOPAA structure. **Thus this study lays the foundations for the optimization enzyme and potential biotechnological application.**

This study proved the dimerization of OPAA via several independent measurements with amOPAA, the phOPAA wild type and its mutants.

The three-dimensional structures of the complexes formed between enzyme **aC22-β-gal and three types of various ligands** (D-galactonolactone, β-D-galactose and IPTG) have been solved and refined. Interactions of particular ligands with aC22-β-gal have been analyzed and compared in all structures. These interactions have been also compared to interactions of these types of ligands with ec-β-gal.

The presence of the shallow binding mode was not expected in aC22-β-gal contrary to the presumable presence of the deep binding mode. **Complex aC22-β-gal-IPTG is an evidence of the presence of the shallow binding position in aC22-β-gal**. It was experimentally proved that presence of Trp999 [21] not necessary for maintaining of the shallow binding mode in aC22-β-gal.

The molecules of D-galactonolactone and β-D-galactose are bound in the deep binding mode in the active site. Their binding positions and coordinating interactions are very similar. Both of them evoked structure changes in the surrounding of the active site compared to the unliganded enzyme. The binding of D-galactonolactone is accompanied by an enzyme conformational changes in which Phe585 rotates and the side chain of His335 moves up closer to the active site. The observed differences are assumed to contribute to stabilization the transition state in aC22-β-gal.

D-galactonolactone is bound in the 1-5 form which is the less populated isomer according to [23] (the 1-4 form or the alyfatic form are more preferable then in the 1-5 form). **The presence of D-galactonolactone in the active site in the 1-5 form demonstrates the specificity of the enzyme.**

The crystals grew without the ligand in the crystallization condition so the presence of ligands in the active site **proved that the enzyme is capable of ligand binding in the crystal form.** The ligands were bound during the soaking procedure, most probably via channels II [24] in the molecule.

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List of publications and activities

1. Publications

[1] Dušková J., Tishchenko G., Ponomareva E., Šimůnek J., Koppová I., Skálová T., Štěpánková A., Hašek J and Dohnálek J.: Chitinolytic enzymes from bacterium inhabiting human gastrointestinal tract - critical parameters of protein isolation from anaerobic culture. *Acta Biochimica Polonica* **58** (2011) 261–263.

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2. Publication in preparation

[8] Stepankova A., Duskova J., Skalova T., Hasek J., Koval T., Østergaard L.H. and Dohnalek J.: Organophosphorous acid anhydrolase from *Alteromonas macleodii* – a structural study and functional relationship to prolidases.

3. Conference lectures (English):

[9] Štěpánková A., Koval T., Skálová T., Dušková J., Hašek J., Dohnálek J.: Three-dimensional structure of binunctional anhydrolase. Materials structure 18 1a (2011) 28. On: 9th Discussions in Structural Molecular Biology, ČR, Nové Hrady.

[10] Štěpánková A., Koval T., Skálová T., Dušková J., Hašek J., Dohnálek J.: Enzymes capable of organophosphates degradation: a critical review with new structural data. Abstract book of 14th Heart of Europe bio-Crystallography Meeting, Zagan, Polsko (2011).

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[12] Štěpánková A., Skálová T., Dohnálek J., Dušková J., Kolenko. P, Hašek J., Lipovová P.: Structure changes evoked by binding of ligands into the active site of enzyme β-galactosidase from Arthrobacter sp. C2-2. Materials structure 16 1a (2008) b6. On: 7th Discussions in Structural Molecular Biology, ČR, Nové Hrady.

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4. Poster presentations

[14] Štepánková A., Skálová T., Dohnálek J., Dušková J., Kolenko P., Hašek J., Lipovová P.: Complex of β-galactosidase with ligand. Materials structure 17 1a (2010) b54. On 8th Discussions in Structural Molecular Biology, ČR, Nové Hrady.

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[16] A. Štěpánková: X-ray structure analysis of bio-macromolecules active under extreme conditions and their complexes with relevant ligand. On: Workshop ČVUT, ČR, Praha (2009).

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[18] Štěpánková A., Skálová T., Dohnálek J.,Dušková J., Kolenko. P, Hašek J., Lipovová P.: The structures of the cold active enzyme β-galactosidase from *Arthrobacter* sp. C2-2 with three bound ligand. On: The 9th International School on the Crystallography of Biological Macromolecules, Como, Italy (2008).

5. Teaching activities

Courses and seminars on CTU FNSPE:

- Seminar of solid state physics (Seminář teorie pevných látek), 2012
- Thermodynamics and molecular physics (Termika a molekulová fyzika), 2008 – 2009
- Waves, optics and atomic physics (Vlnění, optika a atomová fyzika), 2007 – 2009
- The regular contributions within the several courses of FNSPE.

Supervising the bachelor thesis:

- Jan Stránský: Crystallization of biological macromolecules for X-ray diffraction analysis. CTU, FNSPE, Dept. of Solid state physics. (2009-2010).