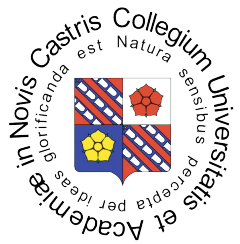

UNIVERSITY OF SOUTH BOHEMIA
Institute of Physical Biology

Ph.D. thesis

Crystallization, structural and modeling studies of photosynthetic membrane protein and water soluble haloalkane dehalogenase

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Nove Hrady
2011

Abbreviations

PSII - photosystem II

$PSII_{CC}$ - PSII Core Complex

PSII RC - PSII reaction centre

OEC - oxygen-evolving complex

3D - three dimensions

X-ray diffraction - Roentgen diffraction

PDB code - protein database code

MD - molecular dynamics

2D - two dimensions

pH - potential of hydrogen

Tris - 2-Amino-2-hydroxymethyl-propane-1,3-diol

PEG - Poly(ethylene glycol)

Asp - Aspartic acid

His - Histidine

Glu - Glutamic acid

Asn - Asparagine

Trp - Tryptophan

Pro - Proline

Gly - Glycine

Thr - Threonine

RMSD - root-mean-square deviation

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Prudnikova, T., 2011: Crystallization, structural and modeling studies of photosynthetic membrane protein and water soluble haloalkane dehalogenase. PhD thesis, in English – 79 p., Institute of Physical Biology, University of South Bohemia, Ceske Budejovice, Czech Republic.

Annotation

This PhD thesis is devoted to study of a) water soluble protein haloalkane dehalogenase DbeA (wild type enzyme) and its mutant variant DbeA1 from bacteria *Bradyrhizobium elkani* USDA94 and also b) the inner membrane protein complex of Photosystem II (PSII) from *Pisum sativum* L.

An active site architecture of the haloalkane dehalogenase enzymes DbeA, DbeA1, stabilization by loop insertion in a mutant form, and structure-function relationships of haloalkane dehalogenases have been studied by using experimental approaches protein crystallization and crystallography. The protein crystals have been grown and X-ray diffraction data on these crystals have been collected and 3D molecular structure has been solved for both dehalogenases DbeA and DbeA1 at 2.2 Å resolution, for DbeA crystals soaked with 1-fluoropentane as a substrate at 1.65 Å resolution, and with 1,3-dichloropropane substrate at 2.15 Å resolution.

Crystallization and molecular modelling methods were applied on membrane protein PSII core complex from higher plants in order to find optimal conditions for the 3D crystals formation, to establish the crystallization protocol and to study light-induced processes in PSII reaction center.

Anotace

Tato disertační práce se zabývá studiem a) ve vodě rozpustného proteinu haloalkan dehalogenasy DbeA (enzym divokého typu) a jeho mutantní variantou DbeA1 z bakterií *Bradyrhizobium elkani* USDA94 a b) membránového komplexu fotosystému II (PSII) z hrachu setého *Pisum sativum* L.

U enzymů DbeA a DbeA1 z rodiny haloalkan dehalogenas byla studována architektura jejich aktivního místa, stabilizace proteinu inzercí peptidové smyčky u mutantní formy a strukturně-funkční vztahy s pomocí experimentálních strukturních metod proteinové krystalizace a krystalografie. V obou případech enzymů DbeA a DbeA1 byly vypěstovány proteinové krystaly a na nich naměřená data z rentgenové difrakce poskytla 3D molekulární struktury s rozlišením 2.20 Å. Krystaly dehalogenasy DbeA saturované substrátem 1-fluoropentanem poskytly 3D strukturu s rozlišením 1.65 Å a v případě substrátu 1,3-dichlorpropanu bylo dosaženo rozlišení 2.15 Å.

Prostřednictvím metod molekulárního modelování a proteinové krystalografie byl také studován membránový protein PSII core komplex z vyšších rostlin.

V rámci krystalizační studie byly hledány optimální krystalizační podmínky pro vznik 3D krystalů PSII a byl vytvořen krystalizační protokol. Modelování na semiempirickém 3D modelu PSII umožnilo studovat světlem indukované procesy ve fotosyntetickém reakčním centru.

Acknowledgements

First of all I would like to thank my supervisors: Michal Kuty and Ivana Kuta Smatanova for their great help, teaching during my PhD study and the thesis preparation. It was so nice to work with them in a kind, peaceful, helpful and patient atmosphere. Their assistance and support allowed this work to materialize. Due to Ivana and Michal I had a possibility to cooperate with many laboratories in Czech Republic, Spain and Germany, to assemble with so interesting people there, to visit structural biology conferences with presentation our scientific results.

My special thanks belong to all members of the Institute of Physical Biology and the head of the Institute of Physical Biology Dalibor Stys for making an immense place to work. I am also very grateful to technical and economical staff of our institute.

I would like to thank Pavlina Rezacova, who taught me all I know about crystallography, and her friendly team (especially Jirka Brynda) for their help during X-ray measurements on the diffractometer, DbeA data processing and very creative discussions.

I also would like to thank our Spanish colleagues from Granada (especially Jose Gavira, Juan Manuel Garcia Ruiz and Estela Pineda Molina), assisted and inspired me a lot during our initial PS II experiments and later work on improvement of PS II isolation and purification.

Although I know that this work would not be possible without biochemical characterization and sample preparation of the haloalkane dehalogenases by our collaboration group from Brno in a person of Jirka Damborsky, Tomas Mozga and Radka Chaloupkova.

I am grateful to Franta Vacha and Ivana Hunalova for PS II sample preparation, assistance and results discussion.

My special thanks belong to all people contributed to my experimental work especially Ivana Tomcova and Peter Palencar for a great help during the first year experiments and especially for a great time spending together; David Kaftan – for consultation and PS II crystallization paper preparation; Ekaterina Sviridova, Oksana Degtjaryk, Vasilina Zayats, Tatsiana Baikova, Volha Shmidt, Hanna Hurina, Alexander Dulebo, Alena Stsiapanava and Mikalai Lapkouski for practical, mental and theoretical support.

Finally, I am very grateful to my family and especially to my husband Uladzimir Prudnikau for his patience, great help and every day support.

Corresponding authors' agreement

Development of crystallization protocol for the DbeA1 variant of novel haloalkane dehalogenase from Bradyrhizobium elkani USDA94. Prudnikova T., Chaloupkova R., Sato Y., Nagata Y., Kuty M., Damborsky, J., Kuta Smatanova I., *Crystal Growth & Design*, **11**, 516-519 (2011)

Toward the crystallization of photosystem II core complex from Pisum sativum L. Prudnikova T., Gavira J. A., Rezacova P., Pineda Molina E., Hunalova I., Sviridova E., Shmidt V., Kohoutova J., Kuty M., Kaftan D., Vacha F., Garcia-Ruiz J. M. and Kuta Smatanova I., *Crystal Growth & Design*, **10**, 3391-3396 (2010)

Crystallization and Preliminary X-ray Analysis of a Novel Haloalkane Dehalogenase DbeA from Bradyrhizobium elkani USDA94. Prudnikova T., Mozga T., Rezacova P., Chaloupková R., Sato Y., Nagata Y., Brynda Y., Kutý M., Damborský J., Kutá-Smatanová I., *Acta crystallographica. Section F*, **F65**(1), 353-356 (2009).

The effects of light-induced reduction of the photosystem II reaction center, a theoretical study. Palencar P., Psencik J., Prudnikova T., Vacha F., Kuty M., *Journal of molecular modeling* **15**(8), 923-933 (2009).

Crystallization and structure-functional study of photosystem II from higher plants. Prudnikova T., Kuty M., Gavira J.A., Palencar P., Vacha F., Rezacova P., Garcia-Ruiz J.M. and Kuta-Smatanova I., *Materials Structure,* **14**(1), 5-7 (2007).

Hereby, I declare, as supervisor and corresponding author of the mentioned papers, that Tatyana Prudnikova, in an association with our colleagues, was responsible for the greater part of the work on crystallographic, modeling and crystallization experiments of the haloalkane dehalogenases DbeA and DbeA1 structural analysis and the photosystem II core complex from higher plants. Tatyana Prudnikova considerably contributed to the data achievement and the lettering of the manuscripts.

Mgr. Michal Kuty, Ph.D.
(Supervisor and co-author of the papers)

Table of content

1. List of publications

I. **Structural Characterization of New Haloalkane Dehalogenase DbeA from Bradyrhizobium elkani USDA94. First HLD with two halide binding sites.** Prudnikova T., Chaloupkova R., Sato Y., Nagata Y., Kuty M., Damborsky, J., Kuta Smatanova I., *Acta crystallographica. Section D* (2011) *in preparation*

II. **Development of crystallization protocol for the DbeA1 variant of novel haloalkane dehalogenase from Bradyrhizobium elkani USDA94.** Prudnikova T., Chaloupkova R., Sato Y., Nagata Y., Kuty M., Damborsky, J., Kuta Smatanova I., *Crystal Growth & Design*, **11**, 516-519 (2011)

III. **Toward the crystallization of photosystem II core complex from Pisum sativum L.** Prudnikova T., Gavira J. A., Rezacova P., Pineda Molina E., Hunalova I., Sviridova E., Shmidt V., Kohoutova J., Kuty M., Kaftan D., Vacha F., Garcia-Ruiz J. M. and Kuta Smatanova I., *Crystal Growth & Design*, **10**, 3391-3396 (2010)

IV. **Crystallization and Preliminary X-ray Analysis of a Novel Haloalkane Dehalogenase DbeA from Bradyrhizobium elkani USDA94.** Prudnikova T., Mozga T., Rezacova P., Chaloupková R., Sato Y., Nagata Y., Brynda Y., Kutý M., Damborský J., Kutá-Smatanová I., *Acta crystallographica. Section F*, **F65**(1), 353-356 (2009).

V. **The effects of light-induced reduction of the photosystem II reaction center, a theoretical study.** Palencar P., Psencik J., Prudnikova T., Vacha F., Kuty M., *Journal of molecular modeling* **15**(8), 923-933 (2009).

VI. **Crystallization and structure-functional study of photosystem II from higher plants.** Prudnikova T., Kuty M., Gavira J.A., Palencar P., Vacha F., Rezacova P., Garcia-Ruiz J.M. and Kuta-Smatanova I., *Materials Structure,* **14**(1), 5-7 (2007).

2. Prologue

Visualization of biological macromolecules in three-dimensional space could provide us information to understand cell biology at the atomic level. X-ray macromolecular crystallography is significant biophysical method for determining the structure of biomolecules including proteins, nucleic acids, drugs and vitamins. The RCSB Protein Databank (Bernstein *et al.* 1977) contains over 76,000 atomic structures of biomolecules and over 87% of these structures were determined by X-ray diffraction.

X-ray crystallography is routinely used to explore the detailed molecular mechanism of enzymes and to determine how substrate interacts with its protein target and what changes might improve it.

The main goal of macromolecular crystallization is to produce well-ordered crystals that are deficient in impurities and big enough to provide the X-ray diffraction. In case proper crystals are obtained, diffraction data can be collected using X-ray radiation beam.

Using an iterative process of modeling and refinement the resulting diffraction pattern is compared to predicted diffraction form of model structure. Model is refined until its predicted pattern match without radical revision of the structure.

Macromolecular crystallization is very difficult process because of the weak nature of protein crystals and irregularly shaped surfaces, both resulting in large channel formation within every protein crystal. The crystallization process is still empirical and mainly based on trial and error.

To crystallize water soluble proteins (like DbeA and DbeA1 haloalkane dehalogenases from *Bradyrhizobium elkani* USDA94), that can diffract “good enough” to provide a structure, without an established protocol can be time-

consuming and very difficult. The protein purity and its concentration, pH, temperature and precipitant composition are the most important factors that influence production of highly diffracting crystals.

Membrane proteins are large components of the genome and include many proteins with vital biological functions, such as structural proteins, receptors, enzymes and ion channels. Intrinsic membrane proteins such as Photosystem II Core Complex remain challenging to crystallize, as they require detergents and other additives for solubilization during their isolation. These detergents often interfere with crystallization solutions. Moreover, the Photosystem II Core Complex is light sensitive and to minimize the complexes' light induced oxidation, blue and red light have to be avoided during the whole process from purification to crystallization. Instead, a dim green ambient light and a microscope equipped with green light filters have to be used.

Up to now the structure of Photosystem II Core Complex from higher plants was not successfully solved by X-ray crystallography.

This thesis describes experimental results of two main projects: 1. Structure-functional study of Photosystem II Core Complex from *Pisum Sativum* L., and 2. Crystallization and X-ray study of water soluble proteins, haloalkane dehalogenases DbeA and DbeA1 from *Bradyrhizobium elkani* USDA94.

The first project was aimed to:

1. Build the molecular model applicable for molecular dynamics simulations.
2. Describe the light-induced processes in Photosystem II.
3. Find optimal crystallization conditions and establish the crystallization protocol for Photosystem II crystals growing.
4. Define the influence of different physical and chemical parameters on Photosystem II Core Complex crystallization.

The main goals of the second project concerning haloalkane dehalogenases DbeA and DbeA1 from *Bradyrhizobium elkani* USDA94 were:

1. Discovering of the most favorable conditions for crystallization of selected proteins.

2. Comparison of DbeA structure with known and published haloalkane dehalogenases.

3. Explanation on a molecular level of unusual biochemical behavior of haloalkane dehalogenase DbeA.

4. Detail explanation of dehalogenation mechanism on two sample substrates.

5. Importance of unique nine amino acids loop insertion from DbjA in DbeA1 haloalkane dehalogenase.

3. Chapter I: Photosystem II Core Complex

3.1. Introduction

Photosynthesis is one of the most exciting process on the Earth, as is essential for oxygenic phototrophic individuals. Photosynthesis is also the source of the carbon in all the organic compounds within organisms' bodies.

The major role in photosynthetic process belongs to the protein called photosynthetic reaction center containing light-absorbing pigments and lipids.

The reaction center embedded in the thylakoid membrane of cyanobacteria, algae, and green plants (Kamiya & Shen 2003) is part of PSII macromolecular complex which is a water–plastoquinone oxidoreductase performing light-driven oxidation of water, with molecular oxygen releasing. The PSII core complex (PS_{CC}) is composed of at least 20 different protein subunits and the PSII functional mechanism cannot be completely understood without detail structural information of its single components.

Several crystallographic structures of PSII from thermophilic cyanobacteria were solved to the maximum resolution of 0.29 nm (Kuhl *et al.* 2000, Zouni *et al.* 2001, Kamiya & Shen 2003, Ferreira *et al.* 2004, Kern *et al.* 2005, Loll *et al.* 2005). Up to now, no 3D crystal structure of PSII complex from higher plants has been solved at atomic resolution. Most PSII subunits are evolutionarily conserved from cyanobacteria to plants, but highly diverse antenna complexes are found in the peripheral parts of PSII.

The $PSII_{CC}$ from green algae and higher plants contains also oxygen-evolving (OEC) water-splitting complex consisting of three external subunits PsbQ, PsbP, PsbO (Kern *et al.* 2005) whereas in cyanobacteria, they are PsbO, PsbV, and PsbU.

The prosthetic groups involved in electron transfer include chlorophyll, pheophytin, quinone, tyrosine and cluster of manganese, calcium, and chloride ions (Ferreira *et al.* 2004).

The model of PSII macromolecular complex (Palencar *et al.* 2009) prepared for molecular dynamics (MD) simulations is shown in Fig. 1. PSII is consisting of four large subunits called psbA, psbD psbB and psbC, heterodimer of cytochrome b-559 (subunits psbE and psbF), and nine low-molecular weight membrane subunits (psbH, psbI, psbJ, psbK, psbL, psbM, psbT, psbX and psbZ). By using of MD simulations and quantum chemical calculations we have tried to explain experimental results concerning the light-induced processes in PSII RC.

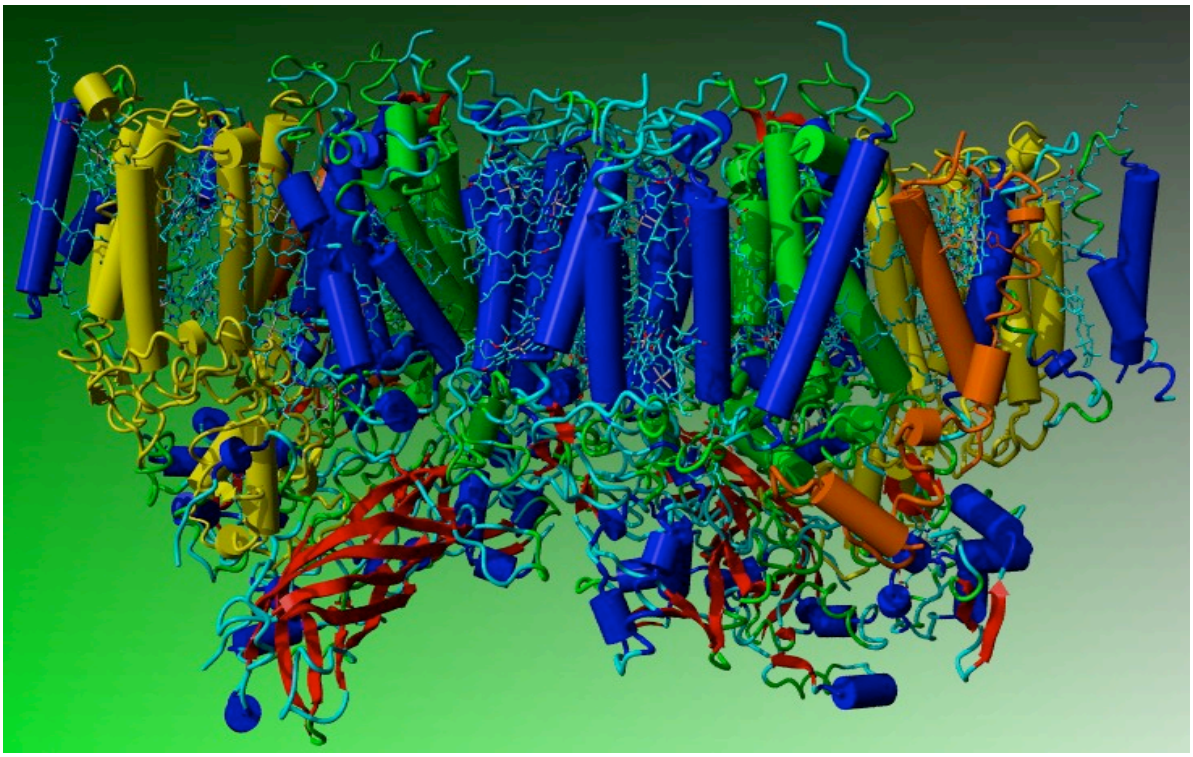

Figure 1. 3D protein model of PSII dimer found in *Thermosynechococcus elongatus* (Loll *et al.* 2005). Front view through the membrane side. Transmembrane helices are represented as cylinders and other protein elements as ribbons, respectively. The model structure is based on X-ray crystal structure obtained from the Protein Data Bank (PDB ID: 2AXT). The model of PSII was constructed by YASARA modeling package (Yasara Biosciences, Krieger *et al.* 2004)).

Crystallization experiments of $PSII_{CC}$ are difficult to perform, particularly because of the complex heterogeneity preventing the proper crystal formation (Ferreira *et al.* 2004). The detergent solubilization as one of the isolation step of sample preparation corresponds to an important parameter affecting the crystal formation (Nugent 1996; Tsiotis *et al.* 1996). Crystallization under green light, which is preventing oxidation of protein solution, makes examination process very difficult as well.

3.2. Experimental methods

For PSII experimental study materials and methods are fully described in presented papers from the list of publications. Furthermore, the initial step of X-ray crystallographic analysis (the data collection approach) is given in this part of PhD thesis.

3.2.1. Crystallographic data collection

At the beginning of the X-ray diffraction experiment the measured crystal has to be mounted on a goniometer head and slowly rotated during X-ray beam. For this purpose, the proper grown monocrystal is usually fishing out from the crystallization drop by a tiny nylon or plastic loop or loading into glass capillaries with the mother liquor (Jeruzalmi 2006). Beyond that the advanced counter-diffusion method (Gavira *et al.* 2006) allowing directly PSII crystal growth in the capillary avoids some moments when crystal can be easily damaged or destroyed. Mounted crystals then have to be flash-frozen with liquid nitrogen. To prevent cracking of crystals during freezing crystals are generally plunged into a cryoprotectant solution (Helliwell 2005).

During the next step, the mounted crystal is placing into an X-ray beam, forming the regular pattern of reflections. These reflections are recorded at every

new position of the crystal, providing the information of the molecular arrangement determination in the crystal (Harp *et al.* 1998). During gradually rotation of the protein crystal, new reflections appear and previous disappear. From the first few images the mosaicity of the crystal and the upper limit on the final resolution of the structure can be determined.

To collect all the necessary data, the crystal has to be rotated gradually through 180°. In the case of the crystal has a higher symmetry, a smaller angular range such as 90° or 45° can be used. Usually the multiple data sets are necessary to solve certain phasing problems (McPherson 2002).

3.3. Results

Papers III and I are describing the step-by-step development of a crystallization protocol from initial screening of large-scale range of physical and chemical parameters to advanced and alternative crystallization techniques.

In the first paper (Crystallization and structure-functional study of photosystem II from higher plants) the crystallization experiments yielding $PSII_{CC}$ quasicrystals and later on needle shape very thin and small crystals were described. These results became the starting point to perform crystals quality improvement, which is published in paper III (Toward the crystallization of photosystem II core complex from *Pisum sativum* L). Therefore, extensive investigation and screening of several precipitants, buffers with pH range 4 - 9, detergents, divalent cations, and cryoprotectants, as well as verification of physical parameters including temperature were applied. The effect was then amended by using of various crystallization methods from standard to advanced and alternative.

Many chemical and physical parameters, which should be controlled in order to get proper protein crystals, have been discussed. The necessity of the use of salt additives, particularly Mn ions (Mn ions have to be presented in both the protein and precipitant solutions) and antioxidants for functionality and stability of the $PSII_{CC}$ sample has been discovered. Unfortunately, pre-soaking experiments during testing of crystal for diffraction measurement caused the crystal cracking. Adding of cryoprotectant directly to the crystallization solution solved this problem. Advanced and alternative crystallization methods provided well-shaped 3D crystals of $PSII_{CC}$ but it was not possible to collect diffraction data set to the resolution necessary for structure determination.

Paper III showed our success in preventing damage of PSII complex by controlling the green light absorption, temperature, additives, co-factors, precipitants even there were several parameters that cannot be controlled. The purified sample was heterogeneous and rather unstable. Therefore, as the most important step before crystallization the quick purification and isolation of $PSII_{CC}$ protein was found out. It was found that it is necessary to focus on discovery a reproducible photosynthetic complex preparation protocol to reduce the number of harmful effects, controlling the level of oxidation and stability of the protein complex.

Paper II (The effects of light-induced reduction of the photosystem II reaction center) was focused on understanding the processes that drives light on RC of PSII complex pigments by MD simulations. For this purpose the 3D model from modified 2axt crystal structure of PSII from *Thermosynechococcus elongatus* (Loll *et al.* 2005) was constructed with reduced form of pheophytin pigment surrounded by D1 subunit of the macromolecular complex. The resulting absorption difference spectra of PSII RC models equilibrated at temperatures of 77

K and 298 K were highly consistent with our previous experiments in which the light-induced bleaching of PSII RC absorbance spectrum was observable only at 298 K. Since the temperature does not influence the excitonic interaction, the temperature dependence of the absorption spectra bleaching upon Pheo reduction does not support the model of Pheo-D1 excitonically interacting with the other chlorins of the PSII RC.

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3.5. Appendix I

Crystallization and structure-functional study of photosystem II from higher plants. Prudnikova T., Kuty M., Gavira J.A., Palencar P., Vacha F., Rezacova P., Garcia-Ruiz J.M. and Kuta-Smatanova I., *Materials Structure,* **14**(1), 5-7 (2007).

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3.6. Appendix II

The effects of light-induced reduction of the photosystem II reaction center. Palencar P., Prudnikova T., Vacha F., Kuty M., *Journal of molecular modeling* **15**(8), 923-933 (2009).

Tatyana Prudnikova: 10% podíl na publikaci

3.7. Appendix III

Toward the crystallization of photosystem II core complex from *Pisum sativum* L. Prudnikova T., Gavira J. A., Rezacova P., Pineda Molina E., Hunalova I., Sviridova E., Shmidt V., Kohoutova J., Kuty M., Kaftan D., Vacha F., Garcia-Ruiz J. M. and Kuta Smatanova I., *Crystal Growth & Design*, **10**, 3391-3396 (2010)

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4. Chapter II: Haloalkane Dehalogenases

4.1. Introduction

Halogenated hydrocarbons are extremely toxic and important class of environmental pollutants, which are commonly used in agriculture and industry. Microbial dehalogenation plays a key role in biodegradation and mineralization processes, providing the enzymatic cleavage of covalent carbon-halogen bond (Kulakova *et al.* 1997). During biodegradation process the halogen atom is substituted by a hydroxyl group, reducing the toxicity of halide and preventing formation of dangerous intermediates like free radicals (D'Arcy *et al.* 2004). The structure-functional characterization of these proteins is important mainly for their industrial relevance. The study of substrate screening of each dehalogenase and affecting the biodegradation reaction rate by application of bioengineering regulation of activity has a potential to construct the universal protecting and treating microbial systems (Prokop *et al.* 2010).

The haloalkane dehalogenases (EC 3.8.1.5) are microbial hydrolytic enzymes of α/β-hydrolase fold superfamily with a two domains organisation: the main and the cap domains. The active site is located between two domains and carries the catalytic triad and two halide-stabilising amino acids. An aspartate as the nucleophile provides the substitution of the halide atom in the substrate (Newman *et al.*1992, Janssen *et al.* 2005).

A novel enzyme haloalkane dehalogenase DbeA, isolated from *Bradyrhizobium elkani* USDA94, was biochemically characterized by Chaloupkova *et al.* (*in preparation*). This enzyme is strongly related to DbjA protein from *Bradyrhizobium japonicum* USDA110 (71% sequence identity) (Sato *et al.* 2005). The DbjA protein carries the unique nine amino acids insertion in the N-terminal part of the cap domain and this nine-amino acid chain has not yet been found in other known haloalkane dehalogenases. DbeA in comparison to DbjA is

generally less active and has a higher specificity towards brominated and iodinated hydrocarbons. These two enzymes also differ in temperature and pH profile (Chaloupkova *et al., in preparation*).

On the base of DbeA enzyme a mutant, named DbeA1, was constructed by inserting the fragment $_{143}$VAEEQDHAE$_{151}$ equivalent to the unique sequence of DbjA between Asp142 and Ala143 of DbeA protein (Sato *et al.* 2007). DbeA1 was formed to study the influence of the insertion in the N-terminus of the cap domain to activity and specificity of DbeA and DbjA haloalkane dehalogenases. Crystallographic analysis of DbeA and DbeA1 protein was applied to reveal the structure-function relationships between these two haloalkane dehalogenases. This insertion may provide an explanation of DbeA1 higher enzymatic activity, different temperature and pH-dependence and other distinctions towards iodinated and brominated aliphatic hydrocarbons.

4.2. Experimental methods

The essential crystallization methods, techniques and materials applied in this work are described in enclosed publications concerning structure-functional study of haloalkane dehalogenases DbeA and DbeA1. Here we are reporting the second and the third step of crystallographic experiment. The first step of crystallographic data collection was already presented in chapter I.

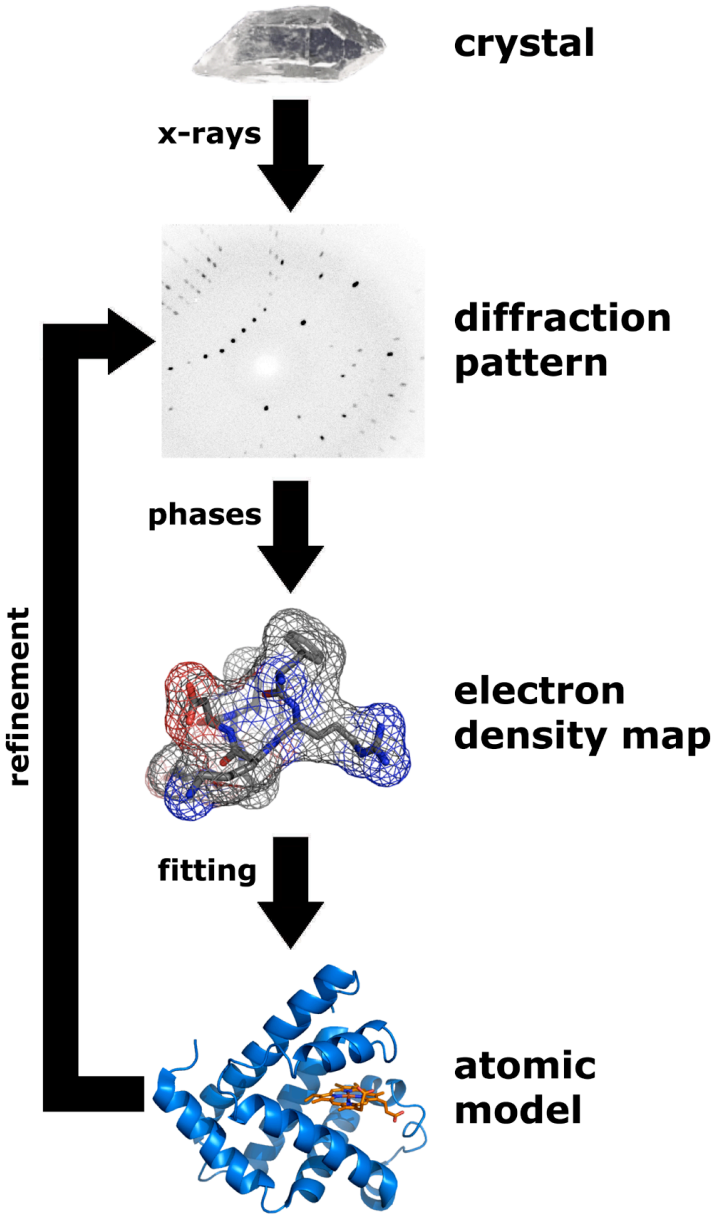

Figure 2. Workflow for solving the structure of a molecule by X-ray crystallography.

(Source: http://en.wikipedia.org/wiki/X-ray_crystallography)

4.2.1. Crystallographic data processing

In the following step the X-ray data are combined computationally, where the two dimensional (2D) images are collected at different rotation angles with corresponding biochemical data, known from the sample, to construct and refine a 3D model of the electron density and later on to arrange atoms within a crystal. This extensive approach could be separated to following parts: indexing, merging, scaling, and phasing.

In the indexing first stage should be determined which variation in the electron density corresponds to which spot on 2D X-ray diffraction images; the relative strengths of the spots in different images (merging and scaling). At the third (phasing) step the total electron density map is formed from the variations (Ravelli & Garman 2006). The resulting electron density is an average of all the molecules within the crystal. Weakly scattering atoms like hydrogen are invisible.

Data processing is usually realized by the reflections autoindexing procedure (Powell 1999). It starts with assignment of the unit cell parameters and identification of every given peak on the X-ray pattern, which corresponds to the correlated position in the reciprocal space. Also, it is necessary to identify the symmetry of the crystal (the space group) during indexing approach and the data then is possible to integrate. This process converting the hundreds of diffraction images, collected at different orientations of the crystal, into a single file with the Miller index and intensity for every reflection, and for each reflection, including error approximation and partiality (Ravelli & Garman 2006).

During images merging process it is possible to recognize which peak appears in two or more images and later on to scale the corresponding image to have a consistent and optimized intensity scale, because the relative intensities of the peaks are essential for the structure determination. At the end it is possible to

evaluate the quality of collected crystallographic data by calculating the symmetry-related R-factor. By this it is possible to identify how many times the symmetry-equivalent reflections were record on the diffraction images and expresses the similarity of measured intensities of symmetry-equivalent reflections (Weiss & Hilgenfeld 1997).

4.2.2. Molecular replacement

The collected diffraction data from protein crystal is a reciprocal space representation of the crystal lattice. The crystal symmetry and the unit cell size and shape are dictating the arrangement of the diffraction reflections during data collection experiment (Hauptman 1997). The intensity of each diffraction reflection is very important because of proportionality to the square of the structure factor amplitude. The structure factor is complex number containing information about the amplitude and phase of the X-ray wave. Both amplitude and phase must be known to construct the electron density map (Wilmanns & Weiss 2005) allows a crystallographer to build a starting model of the molecule.

At the diffraction experiment it is not possible to identify the phase but only the amplitude and this is also called “the phase problem” (Hauptman 1997). Initial phase approximation can be achieved by several ways: molecular replacement, multiple isomorphous replacement, direct methods and anomalous X-ray scattering.

Molecular replacement is the most commonly used method of a phase problem solution. In this approach a related 3D structure has to be known with more then 30% of sequential identity to determine the orientation and position of the molecules within the unit cell (Taylor 2003). The phase of the structure factor of the search model can be used for the refinement. To define the correct

orientation and translation of the chosen model in the unit cell of our crystal the calculation of three rotation and three translation parameters have to be determined (Wilmanns & Weiss 2005).

4.2.3. Model building and refinement

After solution of the phase problem an initial model can be built. This initial model can be used to refine the phases. Every cycle of this process leads to the improvement of the model; therefore a new model would be applied for further structure development. The refinement cycle is fitting atomic positions of the model and their corresponding B-factors (parameter that reflects the thermal motion of the atom) to the observed electron density map, usually yielding a better set of phases (Taylor 2003). The amount of the refinement cycles depends on the difference between the diffraction data and the model, which has to be a minimum. Stereochemistry, hydrogen bonds and allocation of bond lengths and angles are corresponding quantities, which describe the quality of the refined model (Wilmanns & Weiss 2005). But the main parameters that have to be controlled after every round of model improvement are R- and R_{free} factors. Both factors depend on the resolution of the data. R-factor is a criterion of agreement between X-ray diffraction data and constructed crystallographic model, showing how the refined model predicts the measured data. For macromolecules R-factor usually varies from 0.6 to 0.2. R_{free} calculates from a subset of reflections, which were not included to the structure refinement (Brünger 1992).

4.3. Results

The paper I (Crystallization and Preliminary X-ray Analysis of a Novel Haloalkane Dehalogenase DbeA from *Bradyrhizobium elkani* USDA94) is

providing the results of the crystallization and preliminary X-ray analysis of a novel haloalkane dehalogenases DbeA and DbeA1, isolated from *Bradyrhizobium elkani* USDA94. Both the wild type DbeA and the DbeA1 mutant were crystallised using the sitting-drop vapour-diffusion method from the precipitant solution consisting of 100 mM Tris – HCl pH 7.5, 20% (w/v) PEG 3350 or 4000 and 150 mM calcium acetate and 100 mM Tris–HCl buffer pH 7.5, 25% (w/v) PEG 4000 and 130 mM calcium acetate for DbeA and DbeA1, respectively.

DbeA diffraction data were collected at the BESSY beamline MX 14.1 (Berlin, Germany) and, finally, 180 images were used for data processing in HKL-3000 program (Minor *et al.* 2006) with 92 % completeness, 6.6 R_{merge} and 6.0 redundancy. The diffraction data for DbeA1 enzyme were collected at the beamline X11 of the DORIS storage ring with (DESY/EMBL Hamburg, Germany). A complete dataset of 360 diffraction images were processed using the XDS program package (Kabsch 1993), showing 98.9 % completeness, 3.7 redundancy and R_{merge} 12.9. Both crystals were flash-cooled without additional cryoprotection in a liquid nitrogen stream. The crystals of DbeA belong to the primitive orthorhombic space group $P2_12_12_1$, the DbeA1 crystals have the monoclinic space group *C2*. Diffraction data were collected to the resolution of 2.2 Å for both proteins. In one asymmetric unit four DbeA proteins molecules with a ~46 % solvent content and two molecules of the DbeA1 mutant with about 45 % solvent content were situated.

Paper II (Development of crystallization protocol for the DbeA1 variant of novel haloalkane dehalogenase from *Bradyrhizobium elkani* USDA94) was focused on step-by-step protocol building for DbeA1 mutant crystallization for the soaking experiments with two halogenated substrates (1-fluoropentane and 1,3-dichloropropane) to study the active site architecture of the DbeA1 enzyme and

loop insertion stabilization, and to understand structure-function relationships of DbeA and DbjA enzymes.

360 diffraction images for the DbeA1 protein soaked with 1,3-dichloropropane were collected at the beamline X13 of the DORIS storage ring (DESY/EMBL Hamburg, Germany). The X12 beamline (DESY/EMBL Hamburg, Germany) was used for the diffraction measurement of the crystals soaked with 1-fluoropentane with 450 images collection. Both data sets were processed using the HKL-3000 package (Minor *et al.* 2006), reflecting 96.41 % completeness, 6.7 R_{merge} and 4.4 redundancy; and 96.38 % completeness, 5.9 R_{merge} and 3.7 redundancy for DbeA1 crystals, soaked with 1-fluoropentane and 1,3-dichloropropane, respectively.

One – three day old single crystals were used for the soaking experiments at the synchrotron radiation source. Diffraction data sets were collected for 1-fluoropentane at a resolution of 1.65 Å and for 1,3-dichloropropane to 2.15 Å with a soaking time of 24 and 15 hours, respectively. The DbeA1 crystals exhibited *C2* space group. Evaluation of the crystal-packing parameters indicated presence of two molecules of the DbeA1 protein with about 56 % solvent content and a Matthew's coefficient of 2.8.

Paper III (Biochemical and Structural Characterization of New Haloalkane Dehalogenase DbeA from *Bradyrhizobium elkani* USDA94) will illustrate the DbeA crystal structure determination and refinement using diffraction data to 2.2Å resolution. The known structure of the haloalkane dehalogenase from *Rhodococcus sp*. (PDB code: 1bn6; Newman *et al.* 1999) with 51.8 % sequence identity to DbeA, renumbered according to gene sequence, was used as a template for the molecular replacement.

The DbeA structure comparison with DhaA from *Rhodococcus sp*. (1bn6), DmbA from *Mycobacterium tuberculosis* (2qvb), DhlA from *Xanthobacter autotrophicus* (1cij) and LinB from *Sphingomonas paucimobilis* (1cv2) showed a typical α/β hydrolase's fold superfamily two domain organization with the catalytic triad in the active site Asp 103 (nucleophile), His 271 (base) and Glu 127 (acid).

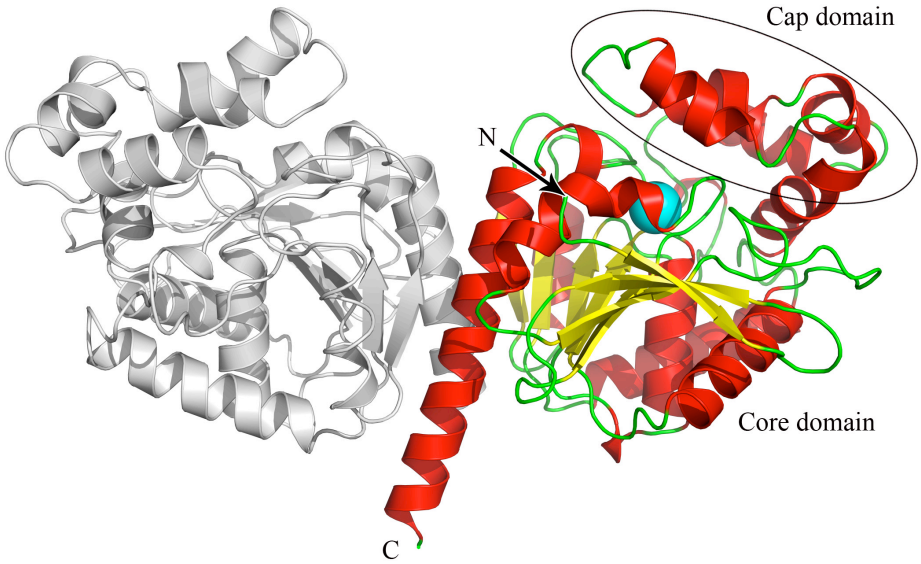

Figure 3. The dimeric structure of DbeA protein.

DbeA is a homodimer in a solution, proved by size exclusion chromatography. The dimerization is realizing through the interaction of the 273–303 residues of C-terminal α-helices. The dimer has two-fold non-crystallographic symmetry with two crystallographic dimers consisting of interacting chain A+D and B+C in the asymmetric unit.

During the refinement, two peaks of electron density were interpreted like two chloride anions. The first chloride anion in DbeA structure occupies typical for all haloalkane dehalogenases product-binding site and interacts with conserved halide binding residues Asn 38 and Trp 104, moreover coordination by Pro 20 and

a water molecule. The DbeA structure has evidently two continuous joint solvent channels to the active site of the proteins: the main tunnel where the active site is situated and so called "slot" at the bottom.

The second chloride anion is situated ~10 Å from the substrate-binding site, hidden deep in the protein core. Side chains of Gln 274, Gln 102, Gly 37 and Thr 40 coordinate it. This chloride-binding site is unique and was not observed in any solved crystal structures of haloalkane dehalogenases. This halide-binding site might have some biological relevance, perhaps for the regulation of DbeA enzymatic activity. This will be proved by biochemical experiments (Chaloupkova *et al.*, *in preparation*).

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4.5. Appendix I

Crystallization and Preliminary X-ray Analysis of a Novel Haloalkane Dehalogenase DbeA from *Bradyrhizobium elkani* USDA94. Prudnikova T., Mozga T., Rezacova P., Chaloupková R., Sato Y., Nagata Y., Brynda Y., Kutý M., Damborský J., Kutá-Smatanová I., *Acta crystallographica. Section F*, **F65**(1), 353-356 (2009).

Tatyana Prudnikova: 60% podíl na publikaci

4.6. Appendix II

Development of crystallization protocol for the DbeA1 variant of novel haloalkane dehalogenase from *Bradyrhizobium elkani* USDA94. Prudnikova T., Chaloupkova R., Sato Y., Nagata Y., Kuty M., Damborsky, J., Kuta Smatanova I., *Crystal Growth & Design*, **11**, 516-519 (2011)

Tatyana Prudnikova: 60% podíl na publikaci

4.7. Appendix III

Biochemical and Structural Characterization of New Haloalkane Dehalogenase DbeA from *Bradyrhizobium elkani* USDA94. Prudnikova T., Rezacova P., Mozga T., Chaloupkova R., Sato Y., Kuty M., Koudelakova T., Nagata Y., Damborsky J., Kuta Smatanova I, *(in preparation)*

Tatyana Prudnikova: 50% podíl na publikaci