Ing. RNDr. Martin Marek, Ph.D., MBA
Loschmidt Laboratories, RECETOX Institute
Faculty of Science, Masaryk University
Kamenice 753/5 Bld. C13, 625 00 Brno, Czech Republic
Phone: 420-5-49498763, Fax: 420-5-49496302
Email: martin.marek@recetox.muni.cz

June 5, 2023

Review of the Doctoral Thesis
of **Andrii Mazur, M.Sc.**

Title: **Crystallographic studies of multimeric and ancestral haloalkane dehalogenases**

Supervisor: **Prof. Mgr. Ivana Kutá Smatanová, Ph.D.**, University of South Bohemia in České Budějovice, Faculty of Science

Introduction

The PhD thesis of Andrii Mazur contains 75 pages. His research focuses on structural characterization of enzymes from a haloalkane dehalogenase (HLD) family. The thesis covers the use of X-ray crystallography for probing structural organization of biomacromolecules, such as proteins and enzymes. Atomic-level knowledge of both the inner organisation and supramolecular complexation of HLDs is crucial for our understanding of their catalytic and non-catalytic functions.

Structure of the thesis

Research motivation, scope, and specific goals of the study are explained in Chapter 1 – Prologue and Specific Aims. The previous works on biochemical and structural characterization of HLD enzymes are summarized in Chapter 2 – Introduction. Special focus is put on structural properties of marine haloalkane dehalogenases and their multimerization potential. Chapter 3 – Materials and Methods is dedicated to the methodology of protein crystallography. The chapter covers methods of protein crystallization, crystallization process optimization, principles of structural organization of crystals and X-ray data diffraction, and strategies to retrieve structural knowledge from diffraction patterns. Chapter 4 – Results and Discussion contains results describing structure determinations of three HLD enzymes, (i) a haloalkane dehalogenase DpaA from *Paraglaciecola agarilytica*, (ii) a lab-constructed putative ancestral $Anc^{LinB\text{-}DmbA}$ enzyme, and (iii) a mutant of Dbe dehalogenase from *Bradyrhizobium elkanii* with an eliminated secondary halide-binding site. The chapter is composed of three original research articles describing the structural characterization of the above mentioned haloalkane dehalogenase enzymes. The impact of gained knowledge for future research directions are explained in Chapter 5 – Conclusions. Finally, literature cited is listed in Chapter 6 – References. The thesis is well structured, following logically topics to receive the results of all specific aims.

Thesis' Review and Questions

The main motivation of the research is that the results will be in great importance for understanding of inner workings and structural organization of HLD enzymes, which are capable to catalyze hydrolytic cleavage of the carbon-halogen bond in various halogenated compounds into corresponding alcohols, halides, and protons. Therefore, the use of HLDs have great potential applications in the detection and degradation of halogenated compounds such as 1,2-dichloroethane, 1,2,3-trichloropropane, 1,2-dibromoethane, which can be interesting for bioremediation and biodegradation applications. Detailed structural knowledge should guide protein engineering efforts to further improve stability, catalytic efficiency and/or specificity of HLD enzymes for their industrial usage.

Towards this aim, Andrii Mazur determined high-resolution crystallographic structures of three haloalkane dehalogenase enzymes. The first structure is a haloalkane dehalogenase DpaA from *Paraglaciecola agarilytica* that exists in a dimeric form in a solution. However, based on the crystal packing, Andrii Mazur and co-workers concluded that the DpaA is tetrameric enzyme, although no experimental data (SAXS) were provided. The second structure was a lab-constructed putative ancestral $Anc^{LinB\text{-}DmbA}$ enzyme, displaying the highest active site cavity volume and the biggest entrance radius on the main tunnel in comparison to descendant enzymes. Finally, the last protein structure determined by Andrii Mazur was a mutant $DbeA^{\Delta Cl}$ with eliminated secondary halide-binding site.

I would like to ask the PhD candidate several following questions:

1. Can you explain why you think that the DpaA is tetrameric enzyme? What is experimental evidence for this statement?

3. Why do you think that the packing observed in the DpaA crystals is unusual?

4. How will your findings contribute to the development of structure-solution procedures for oligomeric proteins that exhibit unusual crystal packing?

5. Can the crystallographic structure of DpaA help to understand the mechanism of enantioselectivity of this enzyme? If so, can you please provide clues for future protein engineering effort to further improve its enantioselectivity?

6. There is a statement that $Anc^{LinB\text{-}DmbA}$ has the highest active site cavity volume and the biggest entrance radius on the main tunnel in comparison to descendant enzymes. Are there any biological reasons why ancestral enzymes displayed voluminous catalytic cavities? And how the opening of the catalytic cavity observed in $Anc^{LinB\text{-}DmbA}$ structure could be affected by either crystal packing and/or a ligand molecule (PEG molecule) bound inside?

7. What is a biological function of a secondary halide-binding site in DbeA dehalogenase? $DbeA^{\Delta Cl}$ is less active and less stable in the presence of chloride salts when compared with the DbeA-wt. Is it common that a catalytic by-product (in this case Cl^-) is stabilising and increasing enzyme activity?

8. Were you able to deduce any allosteric communication pathway between the two halide–binding sites in DbeA? If so, can you provide a broader picture of this unusual regulation for HLD enzymes?

9. I would like to ask the PhD candidate if he can formulate more practical (biotechnological/bioremediation) applications based on achievements in the dissertation.

Summary and Conclusion

The dissertation has contemporary and modern aspect. The topic is very important not only for science but for many practical approaches. The science and industry could use the results achieved in the thesis for future contributions and developments.

The following positive points can be stressed:

1. The research subject is unique for the determination of new crystallographic structures of three haloalkane dehalogenase enzymes.
2. The provided analyses fully correspond to all goals of the thesis.
3. The research topics, the methodology and methods are consistent.
4. The results are derived logically and well described. They are analysed and interpreted in accordance with the scientific standards.
5. The literature references are correctly cited.
6. The thesis clearly demonstrates that Andrii Mazur can conduct independent scientific research and future research work carried out by him independently will meet the standards of his scientific community.

The following points have to be mentioned as potential weakness of the thesis:

1. The major conclusions of the thesis are mainly based on static crystallographic protein structures. I would appreciate to see additional complementary biophysical and biochemical experiments supporting the conclusions. There is limited accompanying functional or mechanistic characterization to decipher the molecular-level mechanisms and functional significance of analysed proteins.
2. Several software tools (Caver, PISA) were used for computational analysis of protein structures. Again, it would be nice to read more critical analysis when interpreting these *in silico* data.
3. There are several inaccuracies in the text, for instance "two tryptophan residues (Asn-Trp)".

This PhD thesis is important and coming in time of multi-disciplinary integrated structural biology. It could be the base point not only for structural biology researchers but also for protein engineers who will be able to improve properties of the studied enzymes. The minor weakness points cannot influence of the final assessment of the thesis results, and I would like to recommend to the scientific committee to award the PhD degree to Andrii Mazur.

Date: June 5, 2023

[redacted]

Ing. RNDr. Martin Marek, Ph.D., MBA