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Evaluation report on the thesis
X-ray Structural Analysis of Flavin- and Cu-Dependent Oxidoreductases for Biotechnological and Medical Purposes
submitted by Ing. Leona Švecová

The doctoral thesis **X-ray Structural Analysis of Flavin- and Cu-Dependent Oxidoreductases for Biotechnological and Medical Purposes** submitted by **Ing. Leona Švecová** presents two subprojects, one studying a flavin-dependent oxidoreductase from *Chaetomium thermophilum* (*Ct*FDO) and a cupper-dependent oxidase from *Myrothecium verrucaria* (*Mv*BOx). These two enzyme are studied in a collaboration with Novozymes A/S. Both enzymes are technologically interesting oxidoreductases with interesting mechanism of catalysis. This fact makes the whole thesis compact.

The Introduction chapter presents the applied biophysical and structural biology techniques and provides an overview of the families of the studied enzymes. My only criticism to this chapter is that it usually does not cite primary source, but I understand this is very difficult for many methods. This is followed by separate Results and Discussion chapters. The results and discussions are really separated, i.e. the discussion does not summarize the results but discuss them in relation to literature and suggests new explanations. Both Results and Discussion chapters contain sub-chapters dedicated to *Ct*FDO and *Mv*BOx.

For *Ct*FDO, the applicant carried out biophysical characterization of the protein, its crystallization and structure determination. As far as I can judge, the structure was determined very carefully. The natural substrate of this enzyme is not known. Therefore the applicant tested a series of potential substrates. Since this effort was not successful, the applicant initiated a high-throughput screening campaign in collaboration with CZ-OPENSCREEN with much larger series of compounds. Also this effort was not successful, so the applicant carried out a crystallographic fragment-based screening. This resulted to identification of binding sites for aromatic moieties and provided a much clearer picture of possible substrates.

For *Mv*BOx, the applicant also characterized, crystallized and determined the 3D structures of the enzyme and its mutants. Again, as far as I can judge, the structure was determined very carefully. An interesting feature of the structure is a covalent adduct of two amino acid residues (Trp and His), which is unique for this enzyme. Site directed mutagenesis in combination with enzymology measurements and structural biology was used to study the role of this adduct.

The thesis is written in English and, as far as I can judge, the level of English is very good. The thesis is nicely illustrated, not only by 3D structure visualization, but also by other types of figures. I especially appreciate the fact that the style of 2D chemical sketches was unified, which is quite rare outside organic chemistry.

I have several question related to *Ct*FDO and *Mv*BOx. My *Ct*FDO questions are:

1. Was only one fragment used in one fragment based screening experiment? It is possible to use a convoluted cocktails of fragments and to deconvolute them for hits. I understand that there was only one ligand in each experiment, but I am bit confused by numbers of screens and datasets.

2. The number of compounds (42) is in my opinion quite low. These compounds were somehow preselected and if yes, how? If not, does it mean that the full screen of ~200 compounds was tested and most of them failed in soaking experiments?
3. An explanation of the failure of HTS campaign suggested by the applicant was that the activity of *Ct*FDO inhibits luciferase assay. Would it be possible to test this?
4. Another approach how to identify the substrate of *Ct*FDO would be to knock out *Ct*FDO in *Ct* and to characterize its phenotype. I understand that this is completely out of scope of the thesis or the specialization of the applicant, but I would ask the applicant whether she is aware of anybody studying this.
My questions related to *Mv*BOx are:
5. Naturally, the most interesting feature of *Mv*BOx is the covalent Trp-His aduct. In the structure published by the applicant there is the bond between Cδ1 of Trp and Nε2 of His (C-N adduct). In principle, the imidazole ring can be flipped to make the bod between Cδ1 of Trp and Cε1 of His (C-C adduct). I understand that participation of the second nitrogen of His in copper ion coordination strongly supports the C-N adduct, but is there also any crystallographic evidence for this type of adduct?
6. The formation of adduct is an oxidative process. Is there anything known about the conditions and the oxidative agent used in this process? Would it be possible to prepare the wild type enzyme without the adduct?
7. Modification of Trp at the Cδ1 position is similar to C-glycosylation of protein. Is there any chemical analogy?
8. The placement of the adduct between the copper ion and ferrocyanide indicates, as pointed out by the author, that the adduct may participate in electron transport. I believe that this would be a nice topic of a separate quantum chemistry and biophysical study. Is there anything like that planned?
9. In my opinion the most famous example of a covalent amino acid aduct in proteins is the one in the green fluorescent protein (GFP). Is there any evidence of fluorescence of the adduct? If yes, would it be possible to use it in research and biotechnology as a protein label, sensor etc.?

Both studies were published in respected scientific journals (*Scientific Reports* and *Acta Crystallographica Section D*) and, despite being very new, they already attracted 5 citations. Corresponding 3D structures were deposited to the Protein Databank (in total 17 structures from the projects covered by the thesis and from another project).

In conclusion, the applicant **Ing. Leona Švecová** has proved her ability to conduct an independent research in the field of structural biology and crystallography and I therefore **fully support granting the Ph.D. degree to the candidate**.

In Úholičky, 17 September 2021

Vojtěch Spiwok