Opponent's Review of the Ph.D. Thesis

Iryna Kishko:
Kinetic behavior of the NAD(P)H: Quinone oxidoreductase WrbA from *Escherichia coli*

The aim of the work of Iryna Kischko was to study enzyme WrbA. This flavoprotein can be found in *E. coli* and belongs to the oxidoreductase family. WrbA catalyzes the oxidation of NADH by a transfer of two electrons; however the physiological role of this enzyme is still unknown. Bigger amount of this protein was prepared in *E. coli* expression system, purified and used for experiments investigating structure of this molecule (including crystallographic experiments) and for enzymatic assays investigating various kinetic parameters.

Ph.D. thesis of Iryna Kishko has 104 pages, and is divided into following parts: Introduction, Methods and Results and Discussion with Conclusions. Each part has its own list of references. The work includes also abstracts of two scientific papers, (one already published, second in the state of submission), which are based mainly on experiments from this Ph.D. thesis. Iryna Kishko is the first author of both of them. The whole thesis is written in English, has a correct graphical form and includes acceptable amount of typing errors. The part Introduction is a good overview which is quite informative.

I think that one of the most important findings reported in this thesis is characterization of the crystal structure of WrbA at very high resolution. This allowed studying detailed conformation of the isoalloxazine ring of the cofactor FMN and revealed so called "propeller twist" conformation. Moreover, the presence of FMN itself was very surprising, since FAD had been used instead of it, during the setting of crystallization experiment. Thus the whole thesis includes very interesting findings and provokes many new questions. On the other hand, it could be written in a better way, in my opinion. I will try to point out some problems I have found and then I will ask some questions about several scientific topics.

Comments:

The description of figures is sometimes quite poor. The Figure III-2 (page 53) has too general legend and bands on the gel seem to be in contradiction with the text on the following page (3 samples tested, the same amount of produced WrbA observed). The description of the Figure III-4 doesn't allow us to connect specific fractions of the elution profile with specific position on SDS-PAGE analysis, values on x and y-axes are not readable, marker of molecular weight is not described, etc.

I have several comments to the measurement of enzyme kinetic. Figures III-14 are redundant, because they are the same as Figures III-15 C, E. (How many times was measured each point?) The first plateau shown on III-15 A, B, C, D is based on one point only. I do not argue about the existence of such a plateau, (shown in much better way on Figure III-13), but if the aim of the experiment is to characterize this type of behavior, it would be nice to measure this region of the curves in detail. The way how

individual points were connected in the Figure III-17 C, seems to me to be too courageous.

Many sentences would need an improvement. I think that sentences like 3rd sentence on page 84, or 3rd and 4th sentence on page 92 would need the improvement very much.

I have these questions:

1. It is written, (page 79), that the measurement was limited by high absorbance of the substrate. Would it be possible to use different wavelength and thus lower the sensitivity of the measurement?

2. Enzyme kinetic has been measured at 5°C, after previous incubation at 5°C, 23°C or 37°C. Longer time of incubation at 23°C (for 48 h or 60 h according to the legend of the Figure III-23) has been used for inactivation of the enzyme. Do you have any hypothesis about the mechanism of such inactivation? What is the temperature optimum for this enzyme and does it correspond with the temperature optimum for *E. coli* growth? Do you think that observed effects could have some physiological relevance?

3. Do you think that the amino acids surrounding FMN in the crystal structure could somehow help to explain the mechanism of enzyme hydrolysis of FAD?

I don't want to diminish the value of this work by previous comments. I think that this work fulfills the requirements for Ph.D. thesis and I recommend it for the defense.

Prague, 5th February 2013

RNDr. Jiří Pavlíček, Ph.D.

Oponentský posudek doktorské disertační práce

Uchazeč(ka): **Mgr. Iryna Kishko**

Název práce: **Kinetic behavior of the NAD(P)H: Quinone oxidoreductase WrbA from *Escherichia coli*.**

The PhD dissertation thesis of Mgr. Iryna Kishko focuses on biochemical and physico-chemical characterization of bacterial flavoprotein WrbA. This enzyme is interesting as it is the founding member of a family of flavodoxin-like proteins and its physiological role in bacteria is still unknown. In order to obtain new information on the enzyme, candidate employed wide range of experimental techniques. Besides the conventional methods of biochemistry and molecular biology, she performed extensive study of enzyme kinetics with respect to three substrates, inhibition studies for several inhibitors/modulators and also succeeded in identification of mechanisms of inhibition by reaction products. Results of kinetic studies suggested presence of two forms of WrbA protein at physiological conditions. This was confirmed by limited proteolysis of WrbA protein and further supported by analytical ultracentrifugation and NMR techniques. Another principal part of the thesis is the protein crystallography. The candidate successfully conducted crystallization experiments obtaining crystals of exceptional quality, that diffracted up to 1.2 Å resolution. The high resolution of the present data allowed conclusive localization of the singly-oxidized state of Met10 side-chain and accurate analysis of the FMN isoalloxazine ring conformation. I personally appreciate that the experimental presented in the thesis are supported by theoretical study. The techniques used for prediction of ligand docking and QM/MM binding energies are described in the section "Experimental Setup" pages 47 - 48, however only brief attention to these calculation is paid in section "Results and Discussion".

The thesis itself is written using classical form and structure, consists of 100 pages of the text without supplements and contain limited amount of typographical and grammatical errors. The presented results are based on two papers published or aimed to distinguished journals. First was published in *PLoS One* and the second is submitted to *Acta Crystallographica*.

Finally, I state that the PhD thesis of Mgr. Iryna Kishko fulfills all criteria for being awarded a PhD degree.

Questions for the defense that should be addressed by the candidate:

1. Is it possible to speculate on possible role of WrbA in bacterial cells?

2. The FMN and FAD cofactors substantially differ in their molecular size and FAD is not incorporated into the WrbA during crystal grow even if present in great excess over FMN. Could you guess how could the FAD interact with the apoform of WrbA and support reduction of benzoquinone?
3. Why were some experiments with WrbA enzymatic activity performed at 5° C?
4. Could you explain the effect of increased ionic strength on initial velocity of WrbA catalyzed reduction of benzoquinone and 2,6-dichlorophenolindophenol (DCPIP), Figure III-18 on page 83?

In Prague, 8. Feb 2013

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Review of the PhD thesis entitled
"Kinetic behavior of the NAD(P)H:Quinone oxidoreductase WrbA from *Escherichia coli*" by Iryna Kishko

The aim of this PhD thesis was to purify WrbA oxidoreductase, determine its structure and elucidate mechanism of its enzymatic action via a detailed characterization of the reaction kinetics. WrbA is an enzyme which belongs to a group of flavin containing oxidoreductases and exhibits a similarity to both the prokaryotic FMN dependent flavodoxins and eukaryotic FAD dependent oxidoreductases. As the exact physiological role of this enzyme remains unknown, elucidation of the mechanism of its action could significantly contribute to the clarification of this role.

PhD thesis of Iryna Kishko represents an example of the „classical" type of the thesis structured into Introduction including aims, Methodology, Results and Discussion, and Conclusions. All these chapters have separate References which is not quite usual but in my view it simplifies searching for the specific references. Introduction (~30 pages) gives an overview on oxidoreductases and their classification, basics of enzyme kinetics and structure of the WrbA protein from E.coli. This chapter also includes aims of the thesis. Chapter II-Methods (~20 pages) is divided into two parts: the first one describes theoretical aspects of procedures and methodologies used in the thesis while the second one describes in detail their practical performance. The question is whether it is necessary to describe theory of generally used methods like electrophoresis, chromatography or absorption spectroscopy in PhD thesis, I think it is appropriate for diploma thesis but not for PhD thesis. The described methods involve molecular biology and biochemical tools used for preparation and purification of WrbA, its structural characterization using limited proteolysis, NMR and crystallization in combination with X ray diffraction analysis, and detailed characterization of the enzyme kinetics. Both chapters demonstrate a good knowledge and overview of Iryna Kishko about the topic of the thesis.

The first part of the Results and discussion section (in summary ~50 pages) describes the overexpression and isolation of WrbA and here (page 54) I found unnecessary repeating of the procedure details which were previously given in the Methods section on page 42. Then the procedures for crystallization of WrbA under various conditions and with various substrates are described and their results shown. I found confusing that on page 57 it is said that efforts to grow crystals of WrbA-FMN were unsuccessful while table III-1 is described as representation of successful conditions for WrbA-FMN crystallization. Here it is certainly worth to mention the finding submitted in a manuscript that although the good crystals were obtained with FAD, the protein within the crystal paradoxically binds FMN. This was revealed by successful X ray diffraction analysis and

obtaining protein structure with the excellent resolution of 1.2 Å. The method of limited proteolysis was then used to assess the oligomeric state of the isolated apo- and holoenzyme in solution. Both dimers and tetramers were suggested to be present in solutions of both proteins with higher level of tetramers in the case of holoenzyme (unlike the occurrence of tetramers in the holoprotein crystals). The presence of both dimeric and tetrameric forms was also speculated as the reason for the occurrence of two plateaus in the dependence of enzyme reaction rate on concentration of substrates. This was further analyzed under various conditions (temperature, pH, presence of modulators) and indirect support for this hypothesis was obtained. Finally, the ping-pond mechanism for the enzyme reaction was proposed based on measurement of kinetics at various fixed concentrations of both substrates, NADH and BQ.

Formally the thesis is written in good English and I found just few unclear English expressions or typing errors which were more frequent at the end of the thesis, for instance on page 92 one sentence is repeated twice. Concerning figures, I found sometimes difficult to understand what they show due to their frequently lower resolution (figures with crystals and molecular structures), small size (figures with plots) and insufficient description and designation. Abbreviations would be good to sort alphabetically for faster searching.

I have few questions:

1. One part of the results presented in the thesis was published in PLOS One journal, another part was included into a manuscript submitted in Acta Crystallographica D, what is the present state of this submission?
2. On page 53, Fig. III-1, the samples of the plasmid contain two bands and there is no designation or description. Is the upper band plasmid with the cloned gene for WrbA and the lower band plasmid without the gene?
3. Can author speculate why WrbA-FMN crystallization was not successful while WrbA-FAD was successful although the structural analysis revealed that the crystallized protein was WrbA-FMN?
4. Does the structure of WrbA allow to theoretically accommodate more bulky cofactor FAD in the binding site for FMN ?
5. It would be worth to correlate the reaction kinetics at a particular salt concentration and oligomeric state of the enzyme as it was performed for temperature dependence by your collaborators. This could be done using limited proteolysis or more simply by native electrophoresis. This would further support the hypothesis about the role of oligomerization in the reaction kinetics of WrbA. Did you perform any experiments in this direction?
6. On page 89 it is said that the membrane-mimicking detergent improves enzyme reactivation but plots in figure III-23 do not confirm it, could you comment on it?

Finally, despite my criticism of several aspects of the submitted thesis, I concluded that the thesis and published paper proved the ability of Iryna Kishko to carry out a good scientific work and I recommend her for a PhD degree.

Josef Komenda

In Třeboň, February 5, 2013 Josef Komenda