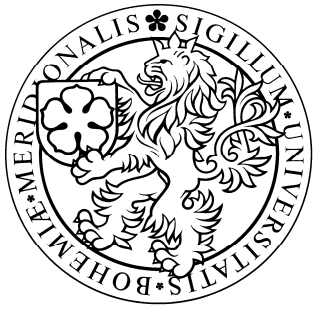
School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Science



Insight into the structure of tetrameric flavoprotein WrbA involved in oxidative-stress response

Ph.D. Thesis

Mgr. Julie Wolfová

Supervisor: Assoc. Prof. Ivana Kutá Smatanová, Ph.D.

School of Complex Systems
Faculty of Fisheries and Protection of Waters
University of South Bohemia in České Budějovice
Zámek 136, 373 33 Nové Hrady
Czech Republic

Institute of Nanobiology and Structural Biology GCRC
Academy of Science of the Czech Republic
Zámek 136, 373 33 Nové Hrady
Czech Republic

České Budějovice 2012

This thesis should be cited as:

Wolfová, J., 2012: Insight into the structure of tetrameric flavoprotein WrbA involved in oxidative-stress response. Ph.D. Thesis, in English. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 2012, pp. 68.

Annotation

This Ph.D. thesis addresses the structural characterization of the unique family of tetrameric flavoproteins WrbA, the role of which in the life of cells is still largely unknown but its enzymatic activity and expression properties implicate it in the cell protection against oxidative stress. Proteins of the WrbA family were proved to carry out two-electron reductions of quinones and in this way to prevent generation of the free radicals, similarly to other flavoproteins known as quinone oxidoreductases. Crystal structures of the liganded and unliganded forms of the prototypical WrbA from *Escherichia coli* were determined. Comparative analyses of these structures with the related flavoproteins were intended to identify and explain the defining structural features of the WrbA family and to clarify its structural and functional relationships to the other flavoproteins.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice,

.....

Julie Wolfová

Financial support

This research was supported by the grants Kontakt ME09016, MSM6007665808 and LC06010 of the Ministry of Education of the Czech Republic and by the grant AV0Z60870520 of the Academy of Sciences of the Czech Republic. Diffraction measurements at the synchrotron DESY/EMBL were supported of the European Community, Research Infrastructure Action under the FP6 "Structuring the European Research Area Specific Programme" to the EMBL Hamburg Outstation, Contract Number RII3-CT-2004-506008.

Acknowledgements

Hereby, I would like to aknowledge everybody who contributed to my progress in theoretical and experimental areas of the structural biology and who helped me to finish this work.

On the first place I am grateful to my supervisor Ivana Kutá Smatanová who introduced me to all the methods of crystallization and allowed me to participate on her projects and to get wonderful experience in protein X-ray crystallography. I appreciate her for being my caretaker and friend all the time even when I wasn't the exemplary student.

I express my thanks to Jannette Carey for hosting me in her laboratory in the Princeton University and for her ideas and writing experience asserted by preparation of the manuscripts.

I would like to thank also to our collaborators Jiří Brynda and Jeroen Mesters for their assistance by X-ray diffraction experiments, data processing and structure solution and for their infinite number of answered questions about the X-ray crystallography and the tricks the crystallographers use.

I should not forget to thank to Michal Kutý and Rudiger Ettrich, who did the great job in the computational chemistry of our protein of interest.

I would like to thank also to Mikalai Lapkouski for his kind company during the X-ray diffraction measurements in Hamburg and for his help in solving the protein structures.

Finally I aknowledge my colleague Tom Garraway for checking my English and also my family for giving me strength, support and all their love when I needed it.

List of papers and supervisor's declaration of author's contribution

The thesis is based on the following papers (listed in the same order as it is in the main text of the thesis):

Paper 1:
Wolfová, J., Grandori, R., Kozma, E., Chatterjee, N., Carey, J., Kutá Smatanová, I. (2005). Crystallization of the flavoprotein WrbA optimized by using additives and gels. *Journal of Crystal Growth* 284 (3-4): 502-505.

Paper 2:
Wolfová, J., Mesters, J.R., Brynda, J., Grandori, R., Natalello, A., Carey, J., Kutá Smatanová, I. (2007). Crystallization and preliminary diffraction analysis of E. coli WrbA in complex with its cofactor flavin mononucleotide. *Acta Cryst*. F63: 571-575.

Paper 3:
Wolfová, J., Brynda, J., Mesters, J.R., Carey, J., Grandori, R., Kutá Smatanová, I. (2008). Crystallographic study of Escherichia coli flavoprotein WrbA, a new NAD(P)H-dependent quinone oxidoreductase. *Materials Structure* 15 (1): 55-57.

Paper 4:
Wolfová, J., Kutá Smatanová, I., Brynda, J., Mesters, J.R., Lapkouski, M., Kutý, M., Natalello, A., Chatterjee, N., Chern, S.Y., Ebbel, E., Ricci, A., Grandori, R., Ettrich, R., Carey, J. (2009). Structural organization of WrbA in apo- and holo-protein crystals. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1794: 1288-1298.

Paper 5:
Carey, J., Brynda, J., **Wolfová, J.**, Grandori, R., Gustavsson, T., Ettrich, R.H., Kutá Smatanová I. (2007). WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases. *Protein Science* 16 (10): 2301-2305.

I hereby declare as the senior and corresponding author of the above mentioned papers, that author of this Ph.D. thesis, Julie Wolfová, is the first author of four papers and co-author of one paper and contributed substantially to these. She carried out most of the experimental work included in the process of crystallization, diffraction data collection, solution of the presented crystal structures and their deposition in the RCSB Protein Data Bank, she performed also the significant part of structural analysis involved in the publications. She did the majority of writing and preparation of tables and figures for papers 1, 2, 3; her role in preparation of manuscript for paper 4 was also significant, including preparation of majority of figures and partial contribution to writing. To paper 5, she contributed mainly with the experimental data.

On behalf of the co-authors, the above-mentioned declaration was confirmed by:

Assoc. Prof. Ivana Kutá Smatanová, Ph.D.
supervisor and co-author of all the papers …….

Abbreviations

3D	three dimensional
WrbA	tryptophan repressor-binding protein A
apoWrbA	unliganded form of WrbA
holoWrbA	WrbA in complex with FMN
TrpR	tryptophan repressor
kDa	kilodalton
DNA	deoxyribonucleic acid
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
NAD(P)H	nicotinamide adenine dinucleotide (phosphate) reduced
Nqo	NAD(P)H:quinone oxidoreductase
Nqo1	mammalian NAD(P)H:quinone oxidoreductase
PDB	Protein Data Bank
PDB ID	the Protein Data Bank identification code
NMR	nuclear magnetic resonance
X-ray	Röntgen radiation
R-factor	factor of reliability
MR	molecular replacement
pH	potential of hydrogen
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol
Trp	tryptophan
Arg	arginine

CONTENTS

APPENDICES

1. INTRODUCTION

The most important role in life is played by molecules of a great complexity, nucleic acids and proteins. Due to their submicroscopic sizes, these molecules cannot be visualized using the common tools for biological research. Using the highly advanced methods to investigate the molecules of life allows scientists to understand properly their structure, function and the processes they participate in. This knowledge can be applied in various areas of human concern like medicine, pharmacy, food production or agriculture. This is the reason why I have chosen structural biology for my postgraduate research. The research addressed in this Ph.D. thesis has been concerned with the investigation of the atomic structure of a protein involved in oxidative stress response. The most common and therefore well-developed method of X-ray crystallography was used in this study. Let me introduce first the reasons for studying the protein structures and then the particular protein studied in this research.

1.1. What can we learn from the protein structure

Proteins are macromolecules crucial for survival of all living organisms. They have evolved to perform a variety of functions. Their different roles, like catalysis, structure building, transport, immune response or cell recognition, are reflected in the range of distinct three-dimensional (3D) structures they can acquire. So far the structures of more than 40 000 proteins and protein/ nucleic acid complexes have been determined, according to the Research Collaboratory of Structural Bioinformatics (RCSB) Protein Data Bank (http://www.pdb.org/, Berman *et al.*, 2000). The essential features of protein structure emerging from studies of a large number of proteins were summarized by Branden and Tooze (1999). The protein molecules are polymers built up from 20 different amino acids linked by peptide bonds to form polypeptide chains. Amino acids along the polypeptide chain interact to form secondary-structure elements known as α helices and β sheets. The spatial arrangement of the secondary-structure elements is called **a protein fold**, and it determines the shape of the functional part of the protein, the active site. The fold ensures that the functionally-important amino acids are brought to proximity to participate on active site formation. The composition and structural arrangement of the active site is therefore crucial for the function of the protein. Proteins of certain functions have a characteristic fold. There is a limited number of possible protein folds due to geometric constraints restricting the spatial arrangement of polypeptide chains. Nowadays the base of the known protein structures

is wide and therefore the chances of finding a truly new fold upon determination of a new protein structure have become low. A new fold hasn't been found since 2009 according to the statistics presented by the RCSB PDB (http://www.pdb.org/, Berman *et al.*, 2000). This means that there is a high probability for the newly-determined protein structure to share the same protein fold with some of the protein structures deposited in the PDB. Based on structural similarity possible functions may be predicted for the protein of which the function is unknown. On the other hand, an unexpected similarity to a known protein fold found for a protein performing some peculiar function may reveal a new functional aspect for this protein fold. Taken together, accumulating of structural data improves our understanding of the structure-functional relationships for the existing protein folds.

Proteins with sequence identity approximately above 30% share the same fold and possibly also the function. They are considered evolutionary related. Together they form **a family of proteins**. Considering the sequence similarity of the proteins sharing the same function and evolutionary origin, it is suggested that the functionally important residues in the protein sequences are most conserved through evolution (Murzin, 1996). The 3D structure of a protein can be predicted by homology modeling. In this method, the protein with known experimental structure serves as a template for building of a structural model of a homologous protein from the same family, of which the structure has not been determined yet (Pandit *et al*., 2004)

Since a large number of protein structures have been uncovered many structural databases have been developed to sort out the protein structures. The database called Structural Classification of Proteins (SCOP) (Murzin et al., 1995) classifies the proteins based on the 3D structure of individual domains they consist of. A domain is a compact globular structure that folds independently and is often associated with a specific function. The single polypeptide chain can fold into one or several domains. Classifying the proteins according to the structural similarities is useful due to the implications of the functional and evolutionary relationships it might indicate.

In summary the investigation of the three-dimensional structure of proteins contributes significantly to understanding of the functional and evolutionary relationships between proteins. The knowledge of protein structure is a prerequisite for understanding of their functions, for learning the mechanisms of their action and the possibilities of their control. Proteins are basic structural and functional constituents of all living organisms which provides many applications of the structural research of

proteins in practical life, e.g. in medicine, drug design, agriculture, food industry or other indusrial branches.

This thesis presents the structural study of a founding member of a recently-discovered family of flavoproteins, WrbA. Based on the sequence similarity with enzymes known as quinone oxidoreductases, the members of WrbA family were suggested and later experimentally confirmed to catalyze two-electron reduction of quinones, which is the mechanism implicating these enzymes in the cell protection against oxidative stress.

1.2. Tetrameric flavoprotein WrbA

1.2.1. First reports on WrbA

The protein investigated in this research is a unique tetrameric flavoprotein WrbA from *Escherichia coli*. It is a small protein, with molecular weight of 21 kDa, built up of 198 amino acids. The first reference to this protein dates back to 1993 when it was reported as a protein co-purifying with the tryptophan repressor protein, TrpR. With reference to its discovery the protein was named tryptophan(W) repressor-binding protein A, WrbA. The interaction of WrbA with TrpR was thought to enhance the stability of DNA binding by TrpR, and thus to have effect on the biosynthesis of tryptophan in prokaryotes (Yang *et al.*, 1993). However, this role for WrbA was put under question, because of no specific effect of WrbA on TrpR-DNA binding was observed (Grandori *et al.*, 1998).

The proper biochemical function of WrbA had not been known for a few years. The role for WrbA was estimated mainly from its sequence analysis and biochemical studies done on the purified protein. Sequence analysis and homology modeling identified *E. coli* WrbA as a prototype of a new family sharing the fold with flavodoxins (Grandori and Carey, 1994). Biochemical studies on *E. coli* WrbA expressed and purified from the recombinant strain of *E. coli* (Grandori *et al.*, 1998) confirmed flavin mononucleotide (FMN) as the physiological cofactor of WrbA. Compared to typical flavodoxins the binding of FMN to WrbA was found to be relatively weak, corresponding to differences in the flavin-binding pocket predicted by homology modeling (Grandori and Carey, 1994). Presence of FMN as a cofactor and similarity to flavodoxins indicated WrbA to be involved in redox reactions.

1.2.2. WrbA is an NADH:quinone oxidoreductase

Due to the efforts made in sequencing and structural genomics, large numbers of protein sequences and the 3D structures of many proteins became available. The number of WrbA homologues increased significantly to show that the WrbA family is widely distributed in living organisms, from bacteria to fungi and higher plants. Phylogenetic analysis revealed strong sequence similarity of the members of WrbA family with the reported NAD(P)H:quinone oxidoreductases (Nqos) (Laskowski *et al.*, 2002; Daher *et al.*, 2005). Some characterized fungal and plant Nqos were even identified as members of the WrbA family, suggesting that WrbA family might share this function. Finally in 2006, Patridge and Ferry experimentally confirmed that *E. coli* WrbA and its homologue in *Archaeoglobus fulgidus* (43% sequence identity to *E. coli* WrbA) display quinone oxidoreductase activity, transferring electrons from NADH as the preferred electron donor to a number of different quinones (Patridge and Ferry, 2006). Enzymes with NAD(P)H:quinone oxidoreductase activity are proposed to maintain quinones in fully reduced state in order to protect cells against harmful oxygen radicals arising from one-electron redox-cycling (Jensen *et al.*, 2002; Cohen *et al.*, 2004; Jaiswal, 2000; Morre, 2004; Wang *et al.*, 2006; Gonzalez *et al.*, 2005; Talalay and Dinkova-Kostova, 2004; Bianchet *et al.*, 2004; Ross and Siegel, 2004). Correspondingly to the suggested cell-protective mechanism *via* the full reduction of quinones, *E. coli* WrbA was shown to transport two electrons at a time resulting in the full reduction of the quinone acceptor (Nöll *et al.*, 2006). The proposed antioxidant role of WrbA is also supported by the results of expression studies showing that transcription of WrbA is controled by the stress response gene *rpoS* (Lacour and Landini, 2004) and a wide range of external stressors, such as acids or H_20_2 induce WrbA's expression (Chang *et al.*, 2002; Tucker *et al.*, 2002, Kang *et al.*, 2005). Based on the given evidence the quinone reductase activity can be considered as a common feature of WrbA family involving its members in the oxidative-stress response.

1.2.3. Unique features of WrbA family

E. coli WrbA was the first characterized member of the WrbA family. According to sequence analysis, homology modeling and biochemical studies, *E. coli* WrbA and its homologues possess several features distinct from typical flavodoxins. The sequences of WrbAs display insertions to the core sequence of flavodoxins predicted to form additional secondary-structure elements (Grandori and Carey, 1994). Unlike

flavodoxins, WrbAs are capable to form dimers and tetramers in solution. The structural elements additional to the flavodoxin fold have been suggested to form a unique subdomain responsible for tetramerization. (Grandori *et al.*, 1998, Natalello *et al.*, 2007). The last distinctive feature is related to the cofactor, FMN. FMN binds to WrbAs much weaker than to flavodoxins, which reflects on the differences between their FMN-binding sites. Considering the oxidoreductase activity of WrbAs, FMN is involved in different reaction mechanism in WrbAs than in flavodoxins. Flavodoxins use FMN for the single-electron transport to protein partners whereas in WrbA, FMN is involved in the two-electron reduction of quinones. The eukaryotic Nqos use different flavin cofactor, FAD, for the two-electron transport. Some significant effects of FMN on the WrbA structure has been shown as well. FMN binding seems to be connected with global reduction in protein dynamics and shift of the subunit assembly equilibrium towards tetramers, as shown by Natalello *et al.* (2007). FMN has been found as a factor improving crystallizability of *E. coli* WrbA (**Paper 2**: Wolfova *et al.*, 2007), indicating the influence of FMN binding on the WrbA structure. Understanding of the unique features of WrbA family with respect to its molecular structure has inspired the pursuit of the crystal structures of WrbA.

1.2.4. Structural information on WrbA family

A number of crystal structures of WrbA from different bacteria have been deposited in the RCSB PDB (http://www.pdb.org/, Berman *et al.*, 2000). However, the structural analyses have been published only for three representatives of WrbA family: WrbA from *Deinococcus radiodurans* , WrbA from *Pseudomonas aeruginosa* (Gorman and Shapiro, 2005) and WrbA from *Escherichia coli* (Andrade *et al.*, 2007; Carey *et al.*, 2007; Wolfova *et al.*, 2009). These structural investigations have confirmed that the WrbA monomers share the fold with flavodoxins. The central fold consists of a central five-stranded parallel β-sheet sandwiched by five α–helices, which is generally known as an α/β open-sheet fold. A binding site for FMN is located in-between the three loops at the carboxy-edge of the β-sheet. Each loop participates in binding of one part of FMN, the isoalloxazine ring, the ribityl and the phosphate group. Two additional structural elements, each of them forming a loop containing one α-helix have been found flanking the core flavodoxin-like fold. These additions correspond to sequence insertions proposed to be unique for the WrbA family. The WrbA monomers have been found to form homotetramers that bind one FMN molecule per monomer, so each

tetramer contains four independent FMN-binding sites. Tetramer has been suggested as the obligatory functional assembly as residues from the three neighbouring subunits cooperate to form the FMN-binding site (see Fig. 2.1).

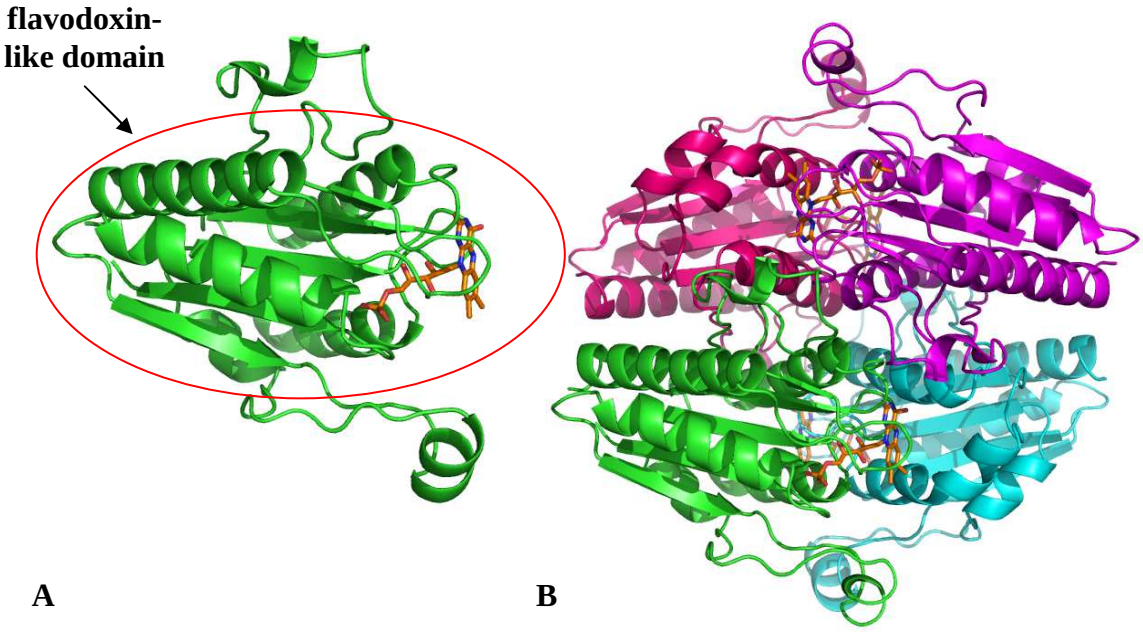


Figure 2.1. Structural models of WrbA from *E. coli* (PDB ID: 2R96, Wolfova *et al.*, 2009): A) monomer; B) tetramer, FMN is shown in sticks.

The first published crystal structures of WrbA from *Deinococcus radiodurans* and *Pseudomonas aeruginosa* (Gorman and Shapiro, 2005) have provided the basic structural information including the main fold of WrbA monomers, FMN-binding interactions and the tetramer assembly. Taking into account that WrbA from *D. radiodurans* displays structural adapatations typical of the extremophilic organisms, and that the structures of WrbA from the mesophilic *P. aeruginosa* are disordered in the segments characteristic of WrbA family, crystal structures of other members of WrbA family have been required to precisely define the unique features of WrbAs and to understand their functional implications. Crystal structures of the prototypical *E. coli* WrbA (Andrade *et al.*, 2007; Carey *et al.*, 2007; Wolfova *et al.*, 2009) have extended the structural knowledge of WrbAs significantly. Three crystal structures of *E. coli* WrbA: two structural models of WrbA in complex with FMN, here referred to as **holoWrbA**, and a structural model of the unliganded form, here referred to as **apoWrbA**, are presented in this thesis as major outcomes of our research (Carey *et al.*, 2007; Wolfova *et al.*, 2009). The structure of apoWrbA was determined at the highest

resolution ever reported for WrbA apoprotein and its structural comparisons with holoWrbA allowed the investigation of the response of the WrbA structure to FMN binding. Extensive comparative analyses of all the WrbA crystal structures with the related flavodoxins have provided the key to the structural explanation of the unique features of WrbA and their contribution to the distinct function of WrbA (**Paper 4**: Wolfova *et al.*, 2009). In Carey *et al.* (2007) (**Paper 5**) the focus has been given to structural comparisons of the active site of *E. coli* holoWrbA with those of the functional homologue mammalian Nqo and flavodoxin in order to clarify the structural accounts for the oxidoreductase activity of WrbAs and suggest the possible mechanism of this function. The functional arrangement of the substrates, NADH and benzoquinone, in the active site of holoWrbA has been proposed. Our suggestions have been supported by the crystal structure of *E. coli* holoWrbA in complex with benzoquinone (PDB ID: 3B6K, Andrade *et al.*, 2007) that have confirmed the position of benzoquinone in the active site proposed by our structural model. These authors have presented the crystal structure of *E. coli* holoWrbA in complex with NADH as well, however this substrate has been found in the non-functional position, leaving our proposed position for NADH in the active site experimentally unproved.

1.2.5. Aims of the structural research on *E. coli* WrbA

The structural research on WrbA from *E. coli* has been inspired by the unique, and largely unknown, standing of this protein among the other flavoproteins. *E. coli* WrbA is a founding member of the novel family of flavoproteins displaying unusual biochemical and functional features with respect to the close-related flavodoxins: tetramerization, weak FMN binding and quinone oxidoreductase activity. At the beginning of our research, structural information available on WrbAs was rather scarce to rationalize the unique features defining the WrbA family and to understand the relationship of WrbAs to other flavoproteins. Therefore our work has been aimed at extending the structural knowledge of this distinctive family of flavoproteins and clarifying its structural and functional relationship to flavodoxins and quinone oxidoreductases. The structural information has been pursued with a perspective to understand the role of WrbAs in the living organisms.

Main goals pursued in this research:

1. Crystallization of the *E. coli* WrbA in both forms – apoWrbA and holoWrbA.
2. Solving the crystal structures based on the X-ray diffraction data collected on the obtained crystals and their deposition into the PDB.
3. Detailed comparative analysis of the crystal structures of apoWrbA and holoWrbA to investigate the effect of FMN binding on the WrbA structure.
4. Structural comparisons of WrbA to flavodoxins in order to identify the structural distinctions and explain them in terms of molecular structure and function.
5. Structural comparisons of WrbA to its functional homologues, Nqos, in order to shed light on the oxidoreductase activity of WrbA in context of its molecular structure and function and to clarify the relationship of WrbAs to Nqos.

2. METHODS USED IN PROTEIN X-RAY CRYSTALLOGRAPHY

Methods used for the research are described well in the published papers, therefore just the fundamentals of the techniques applied in determination of protein structures by X-ray crystallography are introduced in this part. X-ray crystallography has been the most common method used for determination of the three-dimensional structure of proteins so far. Out of the other methods that are included in structural research I should mention the nuclear magnetic resonance spectroscopy (NMR) and electron crystallography. About 90% of the protein structures deposited in the PDB (http://www.pdb.org/, Berman *et al.*, 2000) up to date have been determined by X-ray crystallography. The frequent usage and popularity of this method have contributed to the rapid development of instrumentation and software used for obtaining and handling the X-ray diffraction data, as well as to progress towards automatization of the crystallization procedure.

The crystallographic method consists in diffraction of X-ray beam from the repeating array of many identical molecules - a well-ordered crystal. X-rays interact with electrons in a crystal that deflect them from their incident direction. Therefore X-ray crystallography provides an image of distribution of electrons in a molecule. As electrons occur as electron clouds around atoms, the atomic positions can be found by X-ray crystallography. The whole process of determination of the 3D-structure of a molecule can be divided into several stages: Crystallization. Collection of X-ray diffraction data on crystals. Data processing. Structure solution. Structure refinement and model building. Evaluation of quality of structural models.

2.1. Crystallization of proteins

2.1.1. Principle of crystallization

Crystallization is a phase transition by which molecules segregate from the solution to form the solid phase - a crystal. The process of crystallization is usually divided into three steps: nucleation, growth and termination of growth. Nucleation is a process of formation of the stable aggregates of molecules which are the origins of the future crystals. It takes place in the so called supersaturated solutions where the concentration of protein is higher than the solubility limit. Nucleation is a prerequisite for crystal growth – a process of transferring molecules from the solution to a crystal surface. As the crystals grow concentration of protein in solution decreases until the

concentration of protein reaches the solubility value, what means protein in solution is in equilibrium with its crystalline form and the crystal growth stops.

Quality of the crystals depends on their growth rate. Growth of crystals at high supersaturation results in less ordered crystals, higher defect density and other problems that degrade the quality of crystals. Principle used for growing crystals can be demonstrated on the phase diagram (see Fig. 2.2). The solubility curve (saturation limit) of a protein divides the phase diagram into the regions of undersaturation and supersaturation. There are several different zones in the supersaturated region – nucleation can occur only in the zone with the sufficiently high supersaturation. The higher the supersaturation the more nuclei form and the faster the crystals grow, resulting in a plenty of small crystals which are not suitable for X-ray diffraction analysis. The slower crystal growth occurs at the region of the phase diagram called metastable zone, where a few large, single crystals are formed, but no nucleation occurs. Strategy of the methods used for protein crystallization is to slowly create the lowest level of supersaturation where the nuclei begin to grow and then let the system enter the metastable zone, where a few crystals would continue to develop.

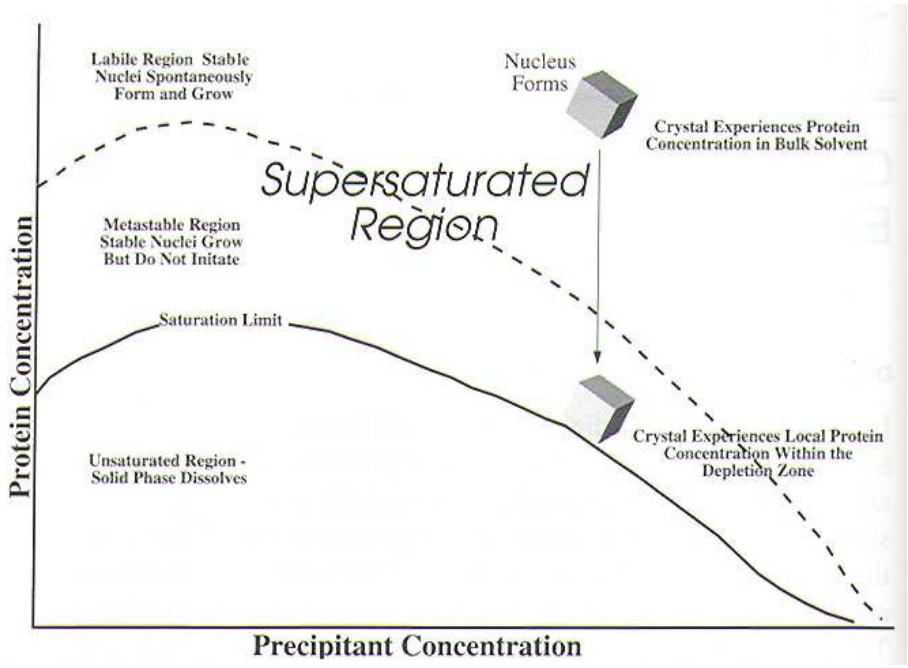


Figure 2.2. Phase diagram of a protein showing different zones in the supersaturation region where nucleation and crystal growth occurs. Adopted from McPherson (1999).

2.1.2. Crystallization methods

There are several approaches to create supersaturation in protein solutions, generally a precipitant agent such as salt, some organic solvent or polymer is introduced into the protein solution, reducing the solubility of proteins and eventually causing formation of protein crystals. Changes of temperature and pH, parameters to which proteins are sensitive, can affect the protein solubility as well. The rate of achieving supersaturation influences the size and quality of protein crystals and therefore it must be controlled. Several methods have been developed to implement the strategy for growth of good-quality protein crystals, that means achievement of low supersaturation values at sufficiently slow rates to induce formation of a few larger and well-ordered crystals. These methods are based on temperature changes (thermal methods), evaporation of the solvent (vapour diffusion methods) and slow mixing of solutions either through a membrane (dialysis) or through free liquid interface (counter-diffusion methods) (McPherson, 1999). I will outline only the methods used in this work, which are vapour diffusion methods representing the standard techniques and counter-diffusion methods considered as advanced techniques.

2.1.2.1. Vapour diffusion methods

Methods based on vapour diffusion are the most popular methods used by protein crystallographers. In these crystallization methods the supersaturated protein solution is created by combining the effect of the precipitation agent with evaporation of solvent from the protein solution. They are performed in the closed systems, where a droplet containing the buffered protein solution and precipitating agent is set up against a reservoir containing a solution of precipitating agent at higher concentration than at the droplet. The difference in precipitating agent concentration between the drop and the reservoir solution causes water to evaporate from the drop and the concentration of protein is increasing until equilibrium is reached and the concentrations of the precipitant in the drop and in the reservoir are equal. These mechanisms can be applied in different geometric arrangements, mostly used are hanging drops or sitting drops (see Fig.2.3). An advantage of these methods is the easy control of the kintetics of crystallization by changing the rate of water evaporation.

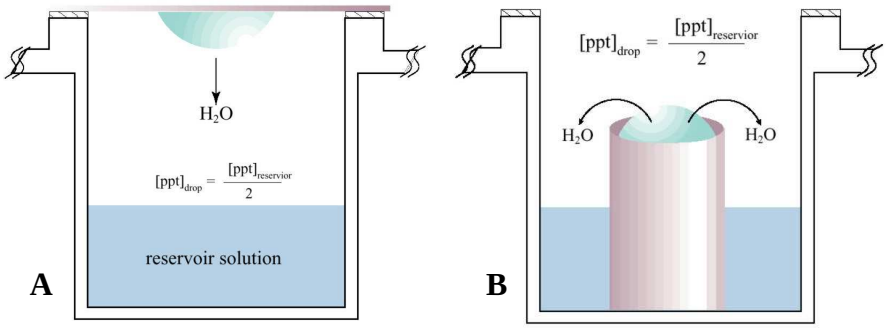


Figure 2.3. Experimental set-up of vapour diffusion in A) a hanging drop, B) a sitting drop. [ppt] denotes concentration of the precipitant. Adopted from McPherson (1999).

2.1.2.2. Counter-diffusion methods

Counter-diffusion methods are known as the advanced techniques for crystallization because there is a continuous change of supersaturation in space and time applied to achieve crystal growth. In these methods the crystals are attempted to be grown under slow diffusive mass transport, which is known to produce well-ordered crystals. Because of the geometry and mass transport involved, they are called counter-diffusion techniques (García-Ruiz, 1991; García-Ruiz, 2003). The counter-diffusion techniques are arranged such that volumes of the precipitating agent and protein solution are positioned to one another inside the glass capillary either in direct contact or separated by a membrane or by an intermediate chamber (containing gel or liquid) working as physical buffer. The two solutions are set to diffuse against each other, resulting in a gradient of supersaturation along the length of the capillary. The gradient ensures that crystals growing at different points of the capillary do it under a different supersaturation values and different rate of change of supersaturation, so increasing the probability to reach the supersaturation where the well ordered single crystals grow (see Fig. 2.4).

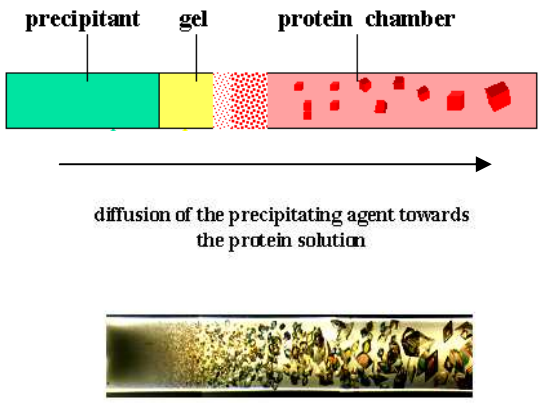


Figure 2.4. Protein crystallization by counter-diffusion method in capillary. Picture at the bottom shows an actual thaumatin crystallization experiment using counter-diffusion method (García-Ruiz, 1991).

This arrangement serves also as automatic screening reducing the number of crystallization experiments (García-Ruiz et al., 2001). Additional implementation of gels in these methods have been found successful in improving quality of the crystals as the gels form environment that may reduce convection and allow the crystal growth under the slow diffusive mass transport (García-Ruiz, 1991; Gavira and García-Ruiz, 2002). The principles and applications of the counter-diffusion methods were reviewed by Ng et al. (2003).

2.1.3. Screening for crystallization conditions and optimization

Crystallization of proteins is a multiparametric process, and there is no way to predict the suitable conditions for crystal growth. To succeed in getting the crystals one has to screen through all the possible crystallization conditions and improve those in which some success was achieved by systematic changing the parameters affecting the crystal growth, as the type and concentration of the precipitating agent, protein concentration, pH or temperature. Crystallization screening kits based on the reagents successfully used for growing protein crystals have been developed (Jancarik and Kim, 1991) to offer a large number of the crystallization conditions for initial screening.

Crystallization conditions can be further optimized by mixing inorganic and organic precipitating agents, crystal seeding, adding stabilizing agents, additives, glycerol and other small molecules. Seeding is applied to both, screening for initial crystallisation conditions and to increase the size and quality of crystals. During seeding events leading to the spontaneous nucleation are separated from those events that allow a crystal nucleus to grow. Commonly used additives for crystallization of biological macromolecules are small organic molecules (alcohols and sugars), detergents, monovalent salts, divalent and trivalent metals, reducing agents, solubilization agents, diamines, detergents protease inhibitors, also substrate analogues, inhibitors, ligands or some other molecules (Stura, 1999).

2.1.4. Protein crystals

Since proteins are large macromolecules that display very weak interaction properties, the protein crystals differ considerably from the conventional crystals of small organic and inorganic molecules. Protein crystals are generally much smaller, extremely fragile and they have very weak mechanical properties. Most of them display a weakly birefringence, which is a consequence of an anisotropy in the crystal seen along the viewing axis. This property makes the crystals of macromolecules coloured if

seen in polarized light under the steromicroscope. These crystals are characteristic with high content of solvent, for most cases 40-60%. The high solvent content means that small molecules, as ligands, cofactors or dyes, can be diffused into macromolecular crystals simply by their addition to the crystals' mother liquor (McPherson 1999).

2.2. X-ray diffraction on crystals and structure determination

2.2.1. The principles of X-ray diffraction by crystals

Crystal is a symmetrical three-dimensional periodic array of molecules, which consists of the repeating elementary unit called a unit cell to form a crystal lattice. It is defined with dimensions a, b, and c and the angles α, β and γ between them. By applying certain operations of symmetry, all the molecules can be built in the unit cells of the crystal. The elements of symmetry in a unit cell define the so called space group of the crystal, the space groups belong to seven crystal systems (triclinic, monoclinic, orthorombic, trigonal, tetragonal, hexagonal and cubic). Unit cell dimensions and the space group of a crystal is reflected in its diffraction pattern. The knowledge of crystal symmetry is essential by interpreting of the diffraction data (Blow, 2002). A complete reference guide to crystal symmetry is provided in: *International tables for crystallography, Volume A* (Hahn, 1995).

If a parallel beam of X-rays falls on a plane of molecules in crystal lattice, the radiation beams are deflected (scattered) from their original path in many directions. The diffraction occurs if the scattered waves interfere with each other and those being in phase are added together and contribute to a system of symmetrically arrenged spots forming a diffraction pattern on the detector. For each spot the intensity of the diffracted wave is measured. The diffraction on parallel planes of crystal lattice occurs if the so called Bragg's condition is fullfilled:

$$n\lambda = 2d\sin\theta \tag{2.1}$$

where n is the integer, λ is the wavelength of X-rays, d is the spacing between the parallel planes in crystal lattice and θ is the scattering angle (see Fig. 2.5). This condition arises if the scattering angle of the X-ray beam deflected by the lattice planes equals the angle of the incident beam, which is actually the condition for the reflection by using these lattice planes as a mirror. Therefore the diffracted beams are referred to as reflections and the planes, on which the X-rays are diffracted, as reflecting planes. Crystals contain many different sets of planes interacting with X-rays but the diffraction occurs only if these planes are oriented in a specific angle towards the X-ray beam.

Thus, crystals are rotated during the diffraction experiment to bring all the planes to the positions where diffraction occurs (Blow, 2002).

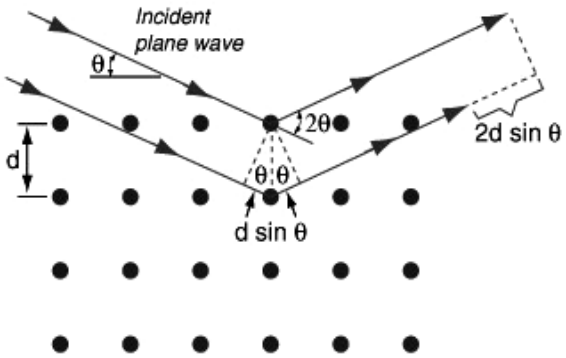


Figure 2.5. Illustration of the Bragg's law. Rays are scattered in a pair of lattice planes separated by a distance d. The path lengths of the rays scattered at the two planes differ by 2d sin θ. Adopted from Thornton and Rex (1993).

2.2.2. Experimental set up and instrumentation

For X-ray data collection protein crystals are mounted in loops or special glass or quartz capillaries and attached to a goniometerhead that allows rotation of the crystal and bringing the planes of the crystals into the position where X-rays are diffracted. The goniometerhead is placed in between the X-ray source and the detector such that the crystal is in the path of X-ray beam. The X-ray source frequently used in laboratories is a rotating anode tube producing characteristic radiation from a copper anode (CuK_{α}, 1.54 Å wavelength). In recent years synchrotron facilities have become widely used as preferred sources for X-ray diffraction measurements. Synchrotron radiation is emitted by electrons or positrons travelling at near light speed in a circular storage ring. The main advantages of synchrotron radiation are high intensity and a wide range of X-ray wavelengths available for the diffraction experiments. High intensity allows data collection on weakly diffracting crystals and also reduces the radiation damage caused to protein crystals by shortening the data acquisition times. Because of radiation damage of the crystals the X-ray pattern dies away after a few hours of exposure at room temperature. To slow down the destructive process and obtain enough data points from X-ray diffraction the crystals must be rapidly frozen and maintained at cryogenic temperatures. This method requires pre-treatment of the crystal with some cryoprotectant to prevent the formation of crystalline ice (López-Jaramillo, 2001; McPherson, 1999). Intensities of the diffracted X-ray beams are measured by X-ray

detectors. The commonly used detectors in these days are image plates and area detectors, such as multiple wire proportional counters or charge coupled devices (CCD).

2.2.3. Processing of X-ray diffraction data

As the crystal is rotated series of diffraction images is taken to record and measure the intensities of all the diffracted waves. Diffraction spots (or reflections) are indexed, that means to each diffraction spot a set of Miller indices (h, k, l) is allocated according to its position in the diffraction pattern and its intensity is on the X-ray detector is recorded. Intensities from all the diffration images are integrated. The possible space groups and the unit cell dimensions are determined and refined throughout the data processing (Leslie and Powell, 2007).

After integration the intensities are merged and scaled. Multiple observations of reflections are merged and average intensities are calculated. Intensities of all the reflections are converted to the amplitudes of the diffracted waves, which are called the structure factor amplitudes. A mathematical relationship is used for this conversion:

$$\left|F_{hkl}\right| = \sqrt{I_{hkl}} \tag{2.2}$$

where $\left|F_{hkl}\right|$ is the structure factor amplitude derived from the intensity I_{hkl} of the reflection at position (h, k, l) of the diffraction pattern. Procedure of French and Wilson (1978) is applied to do the scaling. The best structure factor amplitudes are estimated according to the probability distribution of intensities I_{hkl} (Evans, 2005). These processes are done by using sofisticated software tools developed over many years.

2.2.4. Calculation of the atomic positions from the diffracted waves

The positions of atoms in molecules are determined according to the distribution of electrons in the 3D-space. This is expressed as an electron-density function $\rho(\mathbf{r})$;. here **r** is written for spatial coordinates (*x*,*y*,*z*). A mathematical relationship called Fourier transform is applied to calculate the electron-density function from the amplitude and phase of the diffracted wave. The positions of the diffraction spots are defined with the so called Miller indices (h, k, l) which represent the group of parallel reflecting planes the diffracted wave came from.

All molecules in the crystal unit cell contribute to the total scattering from the unit cell. The total wave diffracted on the parallel reflecting planes of the crystal (one diffraction spot) defined with indices (*h,k,l*) is represented by the structure factor F_{hkl}, a comlex number with the amplitude $\left|F_{hkl}\right|$ and phase θ_{hkl}:

$$F_{hkl} = \left|F_{hkl}\right| \cdot \exp\left[i\theta_{hkl}\right] = V\int \rho(\mathbf{r}) \cdot \exp\left[2\pi i(\mathbf{h}\cdot\mathbf{r})\right]dV \qquad (2.3)$$

The symbol *V* represents the volume of the unit cell, ρ is the density expressed as electrons per unit volume, **h**·**r** is written for an expression: *hx* + *ky* + *lz*; and $2\pi(\mathbf{h}\cdot\mathbf{r})$ is the phase shift at the point (*x, y, z*). The electron-density function is calculated by an inverse Fourier summation over all scattered X-rays and over all reflecting planes (*h k,l*) of the crystal (Blow, 2002):

$$\rho(\mathbf{r}) = \frac{1}{V}\sum_{hkl} F_{hkl} \cdot \exp\left[-2\pi i(\mathbf{h}\cdot\mathbf{r})\right] \qquad (2.4)$$

2.2.5. Structure solution

In this step the electron density function in 3D space (electron density map) is calculated based on the experimental structure factors. Diffraction intensity I_{hkl} is a square of amplitude of structure factor, $\left|F_{hkl}\right|^2$, (see equation 2.2) and thus the amplitudes of diffracted waves can be measured, but there is no information about their phase angles. This is the so called phase problem of crystallography, because both the amplitudes and phases of diffracted waves are needed to calculate the electron-density function (see equations 2.3 and 2.4).

Different methods have been developed to find the phase angles of the reflections and to solve the structure:

- The isomorphous replacement method – heavy atoms are attached to protein molecules in crystals, the phase angles of the intensities and positions of the heavy atoms in the structure are determined first, and these are then used to determine the phase angles and the structure of the protein. Data collection on the native crystals as well as on the crystals of the heavy-atom derivatives is necessary to solve the protein structure.

- The multiple (or single) wavelength anomalous diffraction method – this method requires the presence of sufficiently strong anomalous scattering atoms, e.g. selenomethionine is used in expression of proteins to replace methionine and to introduce the anomalous scattering selenium into the protein structure. The diffraction data collection must be done at a number of X-ray wavelengths, which is one of the disadvantages of this method.
- The molecular replacement method – the phases are determined based on the already known structure which is homologous to the unknown structure.
- Computationally based direct methods – these methods work perfectly only for small molecules and use the phase relations as derived from the probability theory.

All these methods have been well summarized by Drenth (1999). The method used for the solution of the phase problem depends mainly on the data available for the particular protein. Molecular replacement is the most common method applied in macromolecular crystallography and it was the method used in the work presented here. It is why its brief desription is given in the following paragraph.

2.2.5.1. Molecular replacement (MR)

Molecular replacement method is the most frequently used method for structure solution and also the fastest method available. Only one set of data plus the structure of the homologous protein are the requirements for the molecular replacement (MR) method. If some protein of a known structure has a similar amino acid sequence to the unknown protein, it has probably similar tertiary structure. Therefore structure of the homologous protein, referred to as search model, can be used to “lend” the phase angles to the unknown protein. By using the relationship between the electron density function (derived from the protein structure) and X-ray diffraction on this structure (equation 2.3) the structure factor amplitudes and phases can be calculated. The phase angles from the homologous protein are then combined with the experimentally determined structure factor amplitudes and used for finding the first estimate model of the unknown protein. But first, the structure of the homologous protein has to be placed correctly into the unit cell of the crystal of the unknown protein. The proper position and orientation of the search model in the unit cell of the unknown protein is determined in two steps: rotation and translation. In the rotation step the spatial orientation of the known and unknown molecule with respect to each other is determined and in the next step the translation

needed to superimpose the now correctly oriented molecule onto the other molecule is calculated (Drenth, 1999).

Particle repeating in the cell by the symmetry operations is called an asymmetric unit. There can be one or more protein molecules in one asymmetric unit differing in their crystallographic environment and/or conformation. The rotation and translation parameters are usually determined by using only one molecule (monomer) of the search model, but if there are more molecules in the asymmetric unit of the crystal of the unknown protein they can be identified by calculating the so called self-rotation function. This calculation is performed during the rotation search (Navaza, 2001).

If the rotation and translation parameters for placing the search model into the crystal of the unknown protein are found, then the first model of the unknown protein (its electron density function) can be calculated based on the phase angles of the search model and the experimental amplitudes of structure factors determined from the X-ray diffraction data of the unknown protein. The electron-density function in three dimensions, so called electron-density map, can be calculated as an inverse Fourier transform of structure factors F_{hkl} (equations 2.3; 2.4). Nowadays large numbers of the known protein structures increase the chance of finding homologues to the unknown protein, making molecular replacement the most popular method for structure solution.

2.2.6. Structure refinement, model building and evaluation

If all the previous steps of structure determination were successful, the electron-density map obtained after molecular replacement may be interpreted in terms of atomic positions. There are special graphical programs designed for building of the model into the electron density maps, like XtalView (McRee, 1999) or COOT (Emsley and Cowtan, 2004). The quality of the electron-density map depends on the results of all the steps of the structure determination, but the resolution limit of the structure (details that can be seen in the structure) is given by the resolution limit of the crystal. The resolution of the diffraction pattern can be derived from the Bragg's law (equation 2.1):

$$d_{\min} = \frac{\lambda}{2\sin\theta_{\max}} \qquad (2.5)$$

The resolution is taken as the minimal lattice distance, d_{min}, corresponding to the diffraction angle, θ_{max} , at which the reflections are still observed (McPherson, 1999). The resolution depends strongly on the quality of crystals. Crystals whose diffraction pattern extends beyond 2 Å are considered very well ordered (1 Å = 10^{-10} m). Due to the

progress done in all the steps of protein structure determination the majority of the crystal structures entered in the PDB falls into the resolution range of 1 – 2 Å.

After the model of a structure is derived from an electron-density map its accuracy has to be evaluated. Due to the imperfections of the protein crystals and inaccuracies arisen during the data collection, data processing and structure solution, the refinement of the model of a structure is needed. It is the process of adjusting the parameters of the model (bond lengths, bond angles, planarity of bonds) to correspond best to the crystallographic observations. Refinement of the structure is based on minimising the difference between the experimentally determined structure factors and the structure factors calculated according to the model. The agreement index between the observed structure factors and those calculated from the current model is represented by an R-factor. To avoid danger of overfitting the experimental data by the refinement another form of R-factor a free R-factor, R_{free}, has been introduced to assessing the fit of the model to the experimental data (Kleywegt and Brünger, 1996). This free R-factor is calculated from the small set of reflections (5-10%) that have been excluded from the refinement. Cycles of model rebuilding followed by refinement of are repeated till the values of both R and R_{free} are sufficiently low to show a good correspondence of the model with the experimental observations. Reaching values of R below 0.25 and of R_{free} below 0.4 indicates a well-refined structural model according to Brünger (1992).

Another criterion for the accuracy of the structural model are the geometric and stereochemical parameters which should be comparable to the standard values derived from the structures of the small organic molecules and the high-resolution protein structures. The most commonly used program for checking the geometry and stereochemistry of the structures is PROCHECK (Laskowski *et al.*, 1993). Results of the analysis by PROCHECK are required for each new structure deposited into the PDB.

3. OUTLINE OF THE THESIS

In this thesis, the complete structural study of WrbA from *E. coli* done by X-ray crystallography is presented. All the steps involved in the detailed structural analysis of the purified apo- and holoWrbA are described: (1) crystallization, (2) X-ray diffraction on single crystals, (3) structure solution by molecular replacement, (4) model building and refinement, (5) analysis of the 3D structures and comparisons with the structural models of the related proteins.

The thesis is based on 5 published papers in which the results of our structural research on *E. coli* WrbA have been reported:

Paper 1 describes search for crystallization conditions and their subsequent optimization to obtain the first diffraction-quality apoWrbA crystals.

Paper 2 presents crystallization, diffraction analysis and preliminary structure solution of holoWrbA.

Paper 3 is included in this thesis due to the additional details it contains on the newly-grown apoWrbA crystals that were finally used to collect diffraction data and to solve the structure. Improvement of the diffraction properties is demonstrated in this paper.

Paper 4 reports the structure solution and refinement of the two holoWrbA structures and the one of apoWrbA. Extensive comparative analysis of holoWrbA- and apoWrbA crystal structures is involved together with comparisons of all *E. coli* WrbA structures with flavodoxin structures.

In **Paper 5** structural comparison of holoWrbA with mammalian NAD(P)H:quinone oxidoreductase, Nqo1, and flavodoxin, is presented. Relationship of WrbA to Nqo1 and flavodoxin is discussed. Possible mechanism of the oxidoreductase activity of WrbAs is proposed based on the comparative analysis of the active sites and functionally-important residues of WrbA and Nqo1.

4. SUMMARY OF RESULTS AND DISCUSSION

Using a recombinant strain of *E. coli* for expression, *E. coli* WrbA was purified in its apoform, since the cofactor, FMN, was released from the protein during purification due to its weak affinity to WrbA. HoloWrbA was obtained by incubation of the pure apoWrbA with FMN, and so both forms of WrbA were crystallized. The X-ray diffraction data collected on the crystals at synchrotron DESY (Hamburg, Germany) led to the building and refinement of the structural models of both, apo- and holoWrbA. The structural models obtained for WrbA allowed us to investigate the effect of FMN binding on its tertiary and quaternary structure. Comparative analyses of the obtained crystal structures of *E. coli* WrbA with the structures of the related flavodoxins and quinone oxidoreductases were performed to identify the defining structural features of the WrbA family and to find out how they are associated with the unique properties and oxidoreductase activity that have been observed for WrbAs.

4.1. Crystallization and diffraction characteristics of the crystals

Various techniques were applied in crystallization of *E.coli* WrbA , including standard hanging- and sitting-drop vapour diffusion methods, and advanced methods based on counter diffusion. Crystallization turned up to be the most difficult step on the way towards the crystal structures. A number of possible combinations of crystallization conditions were being screened to get the protein crystals of sufficient quality for diffraction analysis.

The first crystals were grown of the apoWrbA by using the common crystallization agents – ammonium sulfate as a precipitating agent and the Tris-HCl buffer of pH 8.5. However, the clusters of plate-like crystals were obtained as the first hit. Such multicrystals couldn’t be used for collection of diffraction data due to the overlapping reflections. Optimization of crystallization by application of additives and gels in the hanging- and sitting-drop arrangements as well as in single capillaries resulted in the first crystals suitable for diffraction experiments. The crystals diffracted X-rays up to a resolution of 2.2 Å when synchrotron radiation was used. The apoWrbA from *E. coli* was the first member of the WrbA protein family, of which the crystals were reported (**Paper 1**).

Crystallization of the holoWrbA appeared to be much easier. The presence of cofactor was found to enhance the crystallizability of the protein significantly. Tetragonal single crystals of the holoWrbA were obtained from several standard

crystallization conditions that were tested in screening through the wide range of conditions. The high-quality crystals were grown from several crystallization conditions by the standard vapour-diffusion technique at 12°C. Unlike apoWrbA crystals, additional optimization steps were not required. Deep yellow colour of the crystals indicated FMN to retain the oxidized state apparent from the absorption spectra taken of the reconstituted holoWrbA before crystallization. The X-ray diffraction experiments revealed two crystal forms differing in unit-cell parameters, space groups and diffraction limits. Using the diffraction data the crystal structures were determined to resolutions of 2.0 and 2.6 Å, which was the highest resolution reported for the holostructures of WrbA homologues available at the time of publication of the *E. coli* holoWrbA crystals and their preliminary diffraction analysis (**Paper 2**).

The positive influence of FMN on crystallization of WrbA stimulated our interest in studying the FMN binding as a factor leading to favorable intermolecular interactions in the WrbA crystals. The crystal structures of both, apoWrbA and holoWrbA, were needed for this investigation. The data collected from the apoWrbA crystals displayed high crystal mosaicity, anisotropy of the diffraction pattern and large unit cell, the features that didn't allow the structure to be solved. Other improvements of crystallization procedure were needed to obtain the apoWrbA crystals with better diffraction properties. Surprisingly, temperature was shown to be another factor playing a significant role in crystallization of WrbA. The apoWrbA crystals were obtained by using the same crystallization conditions as the holoWrbA crystals, except for the temperature. The change of temperature from 12°C to 25°C applied during crystallization allowed the growth of the well-shaped orthorhombic crystals diffracting up to 1.85 Å. The diffraction pattern showed the characteristics of the well-ordered crystal and the complete data set collected on them was successfully used for structure solution (**Paper 3**).

The achievement of the WrbA crystals despite the initial drawbacks confirm that the difficulties of crystallization might be overcome. The work presented here provides several possible solutions of crystallization problems. The crystallization success was accomplished in a number of ways: by using additives and gels in standard and advanced techniques, by co-crystallization with a cofactor and by temperature change during crystallization. Publishing the crystallization reports is important especially when difficulties are experienced because the method applied successfully to overcome them might turn up working well with some other proteins.

4.2. Structure solution and overview of the crystal structures

Diffraction data collected on the reported holo- and apoWrbA crystals were used for structure solution. Data sets obtained on both crystal forms of holoWrbA allowed solution of two structural models of holoWrbA. Self-rotation function together with translation function were applied to the diffraction data to identify the correct space groups. Inspection of the diffraction data by self-rotation function revealed the noncrystallographic twofold symmetry indicating presence of dimers or tetramers in WrbA crystals already in the early stage of the structure solution. Phases for one form of our holoWrbA were determined by molecular replacement using the crystal structure of the holoWrbA from *P. aeruginosa* (PDB ID: 1ZWL; Gorman and Shapiro, 2005) as a template. Structure solution contained a dimer in the asymmetric unit of the crystals. Two dimers were found to form a tetramer with 222 symmetry, consistent with the experimental evidence of tetramerization (Grandori *et al.*, 1998, Natalello *et al.*, 2007). The partially refined structure from the first crystal form of holoWrbA was used as a molecular-replacement template to solve the structure of the other crystal form of holoWrbA. The apoWrbA structure was solved in the same way. All the structures were refined and rebuilt to gain a good R-factor and the final structural models were deposited into the PDB. In summary, there are three crystal structures of *E. coli* WrbA presented in this work: (1) holoWrbA at a resolution of 2.6 Å (PDB ID: 2R96), (2) holoWrbA at a resolution of 2.0 Å (PDB ID: 2R97), (3) apoWrbA at a resolution of 1.85 Å (PDB ID: 2RG1). (The data collection and refinement statistics together with the details of structure solution of both crystal forms of holoWrbA are involved in **Paper 2.** In **Paper 4** the crystal data and structure solution are presented for the later-grown apoWrbA crystals and the final refinement statistics of all three obtained structures is given.)

All the crystal structures were obtained at resolutions sufficient for a proper structural analysis, the structure of apoWrbA was determined to the highest resolution achieved for the apoform of WrbA to date. However, the entire polypeptide chain could be traced for both monomers in the asymmetric unit only in the low-resolution holoWrbA structure. Some segments of the other structures were not resolved, indicating the higher conformational flexibility of the WrbA structures in these segments. Due to this fact, both the holoWrbA structures were used for further analysis to provide the complete structural information.

Detailed analyses of the obtained crystal structures of *E. coli* WrbA are summarised in the following sections. The structures were compared by 3D-superpositions with the long-chain holo- and apoflavodoxin from *Anabaena* (PDB ID: 1FLV, Rao *et al*., 1992; PDB ID: 1FTG, Genzor *et al*., 1996), and with the mammalian NAD(P)H:quinone oxidoreductase Nqo1 (PDB ID: 1QRD, Li *et al.*, 1995). Crystal structure of the short-chain flavodoxin from *Desulfovibrio vulgaris* (PDB ID: 1J8Q, Artali *et al.*, 2002) was used for comparisons of WrbA with flavodoxin in **Paper 5**.

4.3. Overall fold of WrbA and defining features of WrbA

The first view at the crystal structures revealed that in each of the three structures four WrbA monomers form a tetramer, where individual subunits share the common fold of flavodoxins with sequence insertions unique for WrbA family forming additional secondary-structure elements. The corresponding arrangement has been observed in the other reported structures of WrbA (Gorman and Shapiro, 2005; Andrade *et al.*, 2007). Structure of WrbA from *D. radiodurans* displays additional secondary-structure elements to those already mentioned, which is suggested to be the structural adaptation of the protein to resist high radiation experienced by this extremophilic bacterium (Gorman and Shapiro, 2005). The FMN-binding site of apoWrbA isn't empty for ligands as expected, but a small substance identified as chloride ion occupies the pocket where the phosphate group of FMN is bound in holoWrbA. Anion binding into the empty FMN-binding site has been found also in other apoWrbAs (Gorman and Shapiro, 2005) and apoflavodoxins (Martínez-Júlvez *et al.*, 2007; Genzor *et al*., 1996).

The structure-based sequence alignment constructed according to the 3D-superposition of WrbA monomer and flavodoxin has confirmed only one characteristic sequence insertion distinguishing WrbA from flavodoxins, in contrast with previous reports (Grandori and Carey, 1994; Grandori *et al.*, 1998). Insertion interrupting the fifth β-strand aligns with insertion of the long-chain flavodoxins, therefore it was incorrectly assigned as a unique insertion in previous studies. In the long-chain flavodoxins the insertion has been proposed to participate in interactions with protein partners (Peelen et al., 1996; Fromme et al., 2003; Nield et al., 2003). A similar role has been proposed for this insertion in WrbA for it has been found to contact symmetrically-related molecules in the crystals. The insertion assigned correctly as the one characteristic of WrbA family forms a small subdomain that is located together with the equivalent subdomains of the other subunits at the 'poles' of the

WrbA tetramer. This segment provides only a minor contribution to tetramerization in contrast to previous assumptions. However, a new role has been proposed for this unique subdomain in our study. It was suggested to form together with the equivalent subdomain of the neighbouring monomer a channel bringing hydrophobic substrates into the active site of the enzyme *via* the transient membrane interactions. This insertion hasn't been found as a defining feature only in WrbAs. A more extenxive insertion in the same topological position as in WrbAs has been identified also in Nqos (Li *et al.*, 1995), where it forms a small subdomain flanking the flavodoxin fold. This fact indicates the common evolutionary origin of WrbAs and Nqos. (**Paper 4**)

4.4. Structural accounts for tetramerization

WrbA tetramer is formed as assembly of two dimers, with two kinds of interfaces between the neigbouring subunits, a smaller interface involving FMN-binding site, and a larger interface formed across an 'equator' of the tetramer. Detailed analysis of the burried surface areas, residues and interactions occuring at the intersubunit interfaces led us to the conclusion that the dimers are formed preferentially across the larger interface and the tetramers assemble across the smaller FMN-binding interfaces of the pre-formed dimers. This corresponds to the results of the mass spectroscopy that FMN promotes tetramerization while not affecting the monomer-dimer equilibrium (Natalello *et al.*, 2007). Considering oligomerizaion the functional homologue of WrbA, mammalian Nqo1 forms dimer as the active assembly. Two monomers of Nqo1 pair across their FAD-binding surfaces (Li *et al.*, 1995), which is an interaction similar to that formed across the FMN-binding interface of WrbA. Nqo1 can't form tetramers like WrbA due to an additional C-terminal subdomain occupying the position of the possible tetramerization surface (C-terminal subdomains of Nqo1 are located in the position equivalent to the subunits across the larger interface of WrbA tetramer).

Further, analysis of interfaces of the WrbA tetramers indicated that the key elements promoting tetramerization correspond mainly to the integral secondary structure elements occuring also in flavodoxins and not to the unique subdomain of WrbA as proposed earlier (Grandori *et al.*, 1998, Natalello *et al.*, 2007). In order to find out the explanation of tetramerization in WrbAs the tendency to form tetramers was discussed for both, WrbAs and flavodoxins, with regard to hydrophobicity of the surface-exposed areas. The regions of WrbA involved in tetramerization display similar hydrophobicity as the corresponding regions in flavodoxins, but the hydrophobic residues in flavodoxins appear to be shielded from the solvent by the polar functional groups. This result indicated that small changes in the structure of flavodoxins, or its environment, causing exposure of these hydrophobic residues might induce temporary oligomerization in flavodoxins. Consistently with this idea some flavodoxins have

been reported to accumulate dimers (Yoch, 1975; Hoover and Ludwig, 1997). The possibility of dimer formation in other flavodoxins and their functional implications were discussed. (**Paper 4**)

4.5. FMN-induced changes in WrbA and flavodoxin

The effects of FMN on the *E. coli* WrbA structure were shown in all levels of structural organization. The FMN-induced changes in WrbA were revealed from the comparative analysis of holo- and apoWrbA. Potential effects of crystal packing on the structure were distinguished from those of the cofactor binding by proper analysis of crystal contacts in all the structures. FMN binding was observed to have long-range effects, including mainly the arrangement of tetramers, as well as the effects influencing the structure in the vicinity of the FMN-binding site.

Large differences were observed in the arrangement of subunits in WrbA tetramers. These changes were attributed to fixing the loops mediating intersubunit contacts upon FMN binding. Absence of FMN has been found to cause disorder of this loops with the consequence of the substantial changes at the subunit interfaces located at the cores of the tetramers. Apotetramers appeared to lose symmetry, if compared to holotetramers, probably due to the asymmetric increase of the distance between the subunits across the empty FMN-binding site. The long-range effects of FMN binding on WrbA might account for increasing the structural order of WrbA, which could explain the positive influence of FMN on crystallization. Strengthening intersubunit interactions in tetramers upon FMN binding correspond to the results of mass-spectrometry analysis (Natalello *et al.*, 2007) suggesting FMN to favour tetramerization.

Significant structural changes of WrbA structure upon FMN binding are presumably located in the vicinity of the FMN-binding site. Structural overlay of holo- and apoWrbA monomers showed the large displacement of one of the loops contacting FMN in holoWrbA, resulting in partial occupation of the empty FMN-binding pocket in apoWrbA. The bottom of the FMN-binding site, where the chloride ion is bound in apoWrbA, was showen to be almost unaffected by the absence of FMN. The minor effects of the anion on this part of the protein structure indicated the pre-formed anion-binding pocket in apoWrbA.

The 3D-superposition of holo- and apoWrbA with long-chain holo- and apoflavodoxin revealed striking similarities in the behavior of the FMN-binding residues in response to FMN unbinding, despite their different FMN-binding sites. Topologically equivalent residues, Trp57 of flavodoxin and Arg78 of WrbA, swing their side chains by similar angle to

partially occupy the empty FMN-binding site. These shifts have, however, different structural origins and consequences. Whereas in flavodoxin the relative motion of Trp57 is accompanied by the displacement of the entire loop into the empty active site resulting in its tight closure (Genzor *et al*., 1996), the main-chain atoms of apoWrbA Arg78 and the corresponding loop move only slightly relative to holoWrbA due to tetramerization interactions. The shifted Arg78 blocks the empty active site of apoWrbA only partially, though further occlusion occurs by large displacement of one of the neighbouring loops and the other two subunits forming the active site of WrbA. The comparisons of the apo- and holoprotein pairs of WrbA and flavodoxin haven't shown only the similarities and differences of the two proteins in response to FMN binding, but also helped to identify the structurally equivalent residues and their possible roles in the structure. (**Paper 4**)

4.6. Flavin-binding sites of WrbA, flavodoxin and Nqo1

Substantial distinctions between FMN-binding sites of holoWrbA and holoflavodoxin were revealed. The FMN-binding site located at the interface of the three subunits of WrbA tetramer resembles the cavernous pocket consisting of the floor under the isoalloxazine ring presented by one FMN-binding loop and two sides formed from residues presented by the neighbouring subunits. The large binding site was suggested to cause weaker FMN binding. In monomeric flavodoxin, the FMN-binding site is shaped like a slit-like crevice in which the isoalloxazine ring of FMN is tightly bound in between two aromatic residues presented by the two neighbouring loops (Simondsen and Tollin, 1980). Aromatic stacking at the narrow crevice is thought to modulate FMN redox state by stabilizing the semiquinone intermediate thus enabling shuttling of single electrons (Ghisla and Massey, 1989). The mechanism of the two-electron transport promoted by WrbA requires FMN to be fully reduced to its hydroquinone form, which seems to be the favoured FMN redox state in the large FMN-binding site of WrbA (**Paper 5**). According to the analysis of electrostatic potential surfaces, tetramerization and FMN-binding in WrbA were found to be necessary for neutralization of positive charges in the FMN-binding site, in order to allow attraction of the slightly positive substrate, NADH + H^+, into the active site. No corresponding positive charges were shown in the FMN-binding site of flavodoxin (**Paper 4**). The differences found between the FMN-binding sites of WrbA and flavodoxin reflect

the structural adaptations for the different electron-transport mechanisms of the two related proteins.

A surprising structural relationship was found between *E. coli* WrbA and mammalian NAD(P)H:quinone oxidoreductase, Nqo1. The cavernous FMN-binding site of WrbA was found to be similar to that of Nqo1, which analogously to WrbA, promotes two-electron reduction of the electrophilic substrates. Unlike WrbA, Nqo1 is a dimeric protein using FAD for the redox reactions. The mechanism of two-electron transfer catalyzed by Nqo1 requires presence of residues providing a charge-relay pair that promotes hydride transfer (Li *et al.*, 1995). Corresponding residues capable to form the charge-relay pair were found in the FMN-binding site of WrbA. The active site of WrbA was shown to be able to accommodate its substrates, benzoquinone and NADH. Since the active site did not prove to be large enough for simultaneous binding of a cofactor and both substrates, WrbA was predicted to share the characteristic ping-pong kinetic mechanism with Nqo1 (**Paper 5**). Crystal structures of *E. coli* WrbA in complex with benzoquinone and NADH (PDB IDs: 3B6K, 3B6J; Andrade *et al.*, 2007) have confirmed that the molecule of benzoquinone binds to the active site in a similar position to Nqo1. Hence, NADH has been found in a non-functional position providing no additional relevant information.

Based on structural comparisons of the FMN-binding site of WrbA with those of flavodoxin and Nqo1 we could suggest that the assembly of the flavodoxin-like fold into tetramers to form an active site presents an adaptation of WrbA for promoting the two-electron redox reactions.

5. CONCLUSIONS AND PERSPECTIVES

All the steps towards the crystal structures of *E. coli* WrbA were successfully passed through. Detailed analyses of the *E. coli* WrbA crystal structures presented in this thesis have provided significant contributions to understanding of the structural features defining the WrbA family. The structural elements involved in tetramerization were identified by the studying of the tetramer interfaces. Effects of FMN binding on the overall structural ordering, the tetramerization, and the structural arrangement of the active site were described based on the profound comparisons between the holo- and apoWrbA structures. Comparisons with the similar study of the FMN-induced structural changes in flavodoxins revealed surprising similarities. The resembling behaviour in response to FMN binding together with the differences in the FMN-binding sites of WrbA and flavodoxins provided a brighter view on the structural relationship between these two protein families as well as the justification for their functional distinctions. Structural comparisons of the *E. coli* WrbA to mammalian FAD-dependent Nqo1 revealed the close relationship between these proteins and provided hints to understanding the structural features of WrbA in context of its function. The close look into the structures showed how the simple flavodoxin-like fold may be adapted in WrbA to extend the catalytic function from one-electron tranfer between protein partners to two-electron reductions of quinones. WrbA was suggested to be a protein linking bacterial flavodoxins and eukaryotic Nqos in both, structure and biochemical function.

Even though the mechanism of the reactions catalyzed by WrbAs has been suggested, the experimental evidence is still required to confirm our assumptions and to reveal more interesting details of the conditions at the active site when the reaction occurs. Since in the only crystal structure available of *E. coli* holoWrbA in complex with NADH (PDB ID: 3B6J, Andrade *et al.*, 2007) the substrate doesn't bind to a functional position in the active site, crystal structure of holoWrbA with NADH is still to be pursued, as well as the crystal structures of holoWrbA complexed with different kinds of quinones. Our resarch on *E. coli* WrbA has not only extended the structural knowledge base of proteins but it has also provided suggestions of the physiological roles that might serve as inspiration for the future physiological and enzymological investigations.

6. REFERENCES

Andrade, S.L., Patridge, E.V., Ferry, J.G., Einsle, O. (2007). Crystal structure of the NADH:quinone oxidoreductase WrbA from *Escherichia coli*. *J. Bacteriol.* 189: 9101–9107.

Artali, R., Bombieri, G., Meneghetti, F., Gilardi, G., Sadeghi, S.J., Cavazzini, D., Rossi, G.L. (2002). Comparison of the refined crystal structures of wild-type (1.34 A) flavodoxin from Desulfovibrio vulgaris and the S35C mutant (1.44 A) at 100 K. *Acta Crystallogr.* D58: 1787-1792.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E. (2000). The Protein Data Bank. *Nucleic Acids Research* 28: 235-242.

Bianchet, M.A., Faig, M., Amzel, L.M. (2004). Structure and mechanism of NAD[P]H:quinone acceptor oxidoreductases (NQO). *Methods Enzymol.* 382: 144–174.

Blow, D. (2002). Outline of crystallography for biologists. Oxford University Press, Oxford.

Branden, C., Tooze, J. (1999). Introduction to protein structure. Garland Publishing, New York.

Brunger, A.T. (1992). Free R-value: a novel statistical quantity for assessing accuracy of crystal structures. *Nature* 355: 472.

Carey, J., Brynda, J., Wolfová, J., Grandori, R., Gustavsson, T., Ettrich, R.H., Kutá Smatanová I. (2007). WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases. *Protein Science* 16 (10): 2301-2305.

Chang, D.E., Smalley, D.J., Conway, T. (2002). Gene expression profiling of Escherichia coli growth transitions: an expanded stringent response model. *Mol. Microbiol.* 45: 289–306.

Cohen, R., Suzuki, M.R., Hammel, K.E. (2004). Differential stress-induced regulation of two quinone reductases in the brown rot basidiomycete Gloeophyllum trabeum. *Appl. Environ. Microbiol.* 70: 324–331.

Daher, B.S., Venancio, E.J., de Freitas, S.M., Bao, S.N., Vianney, P.V., Andrade, R.V., Dantas, A.S., Soares, C.M., Silva-Pereira, I., Felipe, M.S. (2005). The highly expressed yeast gene pby20 from *Paracoccidioides brasiliensis* encodes a flavodoxin-like protein. *Fungal Genet. Biol.* 42: 434–443.

Drenth, J. (1999). Principles of protein X-ray crystallography. Springer-Verlag, New York, USA.

Emsley, P., Cowtan, K. (2004). Coot: model-building tools for molecular graphics, *Acta Crystallogr.* D60: 2126–2132.

Evans, P.R. (2005). Scaling and assessment of data quality. *Acta Cryst.* D62: 72-82.

French, G.S., Wilson, K.S. (1978). On the treatment of negative intensity observations. *Acta. Cryst.* A34: 517-525.

Fromme, P., Melkozernov, A., Jordan, P., Krauss, N. (2003). Structure and function of photosystem I: interaction with its soluble electron carriers and external antenna systems. *FEBS Lett.* 555: 40–44.

Gavira J. A., García-Ruiz J. M. (2002). Agarose as crystallisation media for proteins II: trapping of gel fibres into the crystals. *Acta Cryst.* D58: 1653 – 1656.

García-Ruiz, J.M. (1991). Uses of crystal growth in gels and other diffusing-reacting systems. *Key Eng. Mater*. 88: 87 – 106.

García-Ruiz, J.M. (2003). Counterdiffusion methods for macromolecular crystallization. *Methods Enzymol.* 368: 130 – 154.

García-Ruiz, J.M., Otálora, F., Novella, M.L., Gavira, J.A., Sauter, C., Vidal, O. (2001). A supersaturation wave of protein crystallization. *J. Cryst. Growth* 232: 149 – 155.

Genzor, C.G., Perales-Alcón, A., Sancho, J., Romero, A. (1996). Closure of a tyrosine/tryptophan aromatic gate leads to a compact fold in apo flavodoxin. *Nat. Struct. Biol.* 3: 329–332.

Ghisla, S., Massey, V. (1989). Mechanismsof flavoprotein-catalyzed reactions. *Eur. J. Biochem.* 181: 1–17.

Gonzalez, C.F., Ackerley, D.F., Lynch, S.V., Matin, A. (2005). ChrR, a soluble quinone reductase of *Pseudomonas putida* that defends against H2O2. *J. Biol. Chem.* 280: 22590–22595.

Gorman, J., Shapiro, L. (2005). Crystal structures of the tryptophan repressor binding protein WrbA and complexes with flavin mononucleotide. *Protein Sci.* 14: 3004–3012.

Grandori, R., Carey, J. (1994). Six new candidate members of the α/β twisted open-sheet family detected by sequence similarity to flavodoxin. *Protein Science* 3: 2185-2193.

Grandori, R., Khalifah, P., Boice, J.A., Fairman, R., Giovanelli, K., Carey, J. (1998). Biochemical characterization of WrbA, founding member of a new family of multimeric flavodoxin-like proteins. *J. Bio. Chem.* 273: 20960-20966.

Hahn, T. (Ed.) (1995). International tables for crystallography. Volume A. Space-group symmetry. International Union of Crystallography/Kluwer, Dordrecht.

Hoover, D.M., Ludwig, M.L. (1997). A flavodoxin that is required for enzyme activation: the structure of oxidized flavodoxin from *Escherichia coli* at 1.8A resolution. *Protein Sci.* 6: 2525–2537.

Jaiswal, A.K. (2000). Regulation of genes encoding NAD(P)H:quinone oxidoreductases. *Free Radic. Biol. Med.* 29: 254–262.

Jancarik, J., Kim, S.H. (1991). Sparse matrix sampling: a screening method for crystallization of proteins. *J. Appl. Cryst.* 24: 409-411.

Jensen, K.A. Jr, Ryan, Z.C., Vanden Wymelenberg, A., Cullen, D., Hammel, K.E. (2002). An NADH:quinone oxidoreductase active during biodegradation by the brown-rot basidiomycete Gloeophyllum trabeum. *Appl. Environ. Microbiol.* 68: 2699–2703.

Kang, Y., Weber, K.D., Qiu, Y., Kiley, P.J., Blattner, F.R. (2005). Genome-wide expression analysis indicates that FNR of Escherichia coli K-12 regulates a large number of genes of unknown function. *J. Bacteriol.* 187: 1135–1160.

Kleywegt, G.J., Brunger, A.T. (1996). Checking your imagination, application of free R value. *Structure* 4: 897-904.

Lacour, S., Landini, P. (2004). SigmaS-dependent gene expression at the onset of stationary phase in Escherichia coli: function of sigmaS-dependent genes and identification of their promoter sequences. *J. Bacteriol.* 186: 7186–7195.

Laskowski, M.J., Dreher, K.A., Gehring, M.A., Abel, S., Gensler, A.L., Sussex, I.M. (2002). FQR1, a novel primary auxin-response gene, encodes a flavin mononucleotide-binding quinone reductase. *Plant Physiol.* 128: 578–590.

Laskowski, R.A., McArthur, M.W., Moss, D.S., Thornton, D.J. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Cryst.* 26: 283.

Leslie, A.G.W., Powell, H.R. (2007). Processing Diffraction Data with Mosflm. *Evolving Methods for Macromolecular Crystallography* 245: 41-51.

Li, R., Bianchet, M.A., Talalay, P., Amzel, L.M. (1995). The three-dimensional structure ofNAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proc. Natl. Acad. Sci. U. S. A.* 92:8846–8850.

López-Jaramillo, J., García-Ruiz, J.M., Gavira, J.A., Otálora, F. (2001). Crystallization and crystallography inside X-ray capillaries. *J. Appl. Cryst.* 34: 365-370.

Martínez-Júlvez, M., Cremades, N., Bueno, M., Pérez-Dorado, I., Maya, C., Cuesta-López, S., Prada, D., Falo, F., Hermoso, J.A., Sancho, J. (2007). Common conformational changes in flavodoxins induced by FMN and anion binding: the structure of *Helicobacter pylori* apoflavodoxin. *Proteins* 69: 581–594.

McPherson, A. (1999). Crystallization of biological macromolecules. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

McRee, D.E. (1999). XtalView/Xfit — a versatile program for manipulating atomic coordinates and electron density. *J. Struct. Biol.* 125: 156–165.

Morre, D.J. (2004). Quinone oxidoreductases of the plasma membrane. *Methods Enzymol.* 378: 179–199.

Murzin, A.G. (1996). Structural classification of proteins: new superfamilies. *Current Opinion in Structural Biology* 6: 386-394.

Murzin, A.G., Brenner, S.E., Hubbard, T., Chothia, C. (1995). SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 247: 536 - 540.

Natalello, A., Doglia, S.M., Carey, J., Grandori, R. (2007). Role of flavin mononucleotide in the thermostability and oligomerization of Escherichia coli stress-defense protein WrbA. *Biochemistry* 46: 543–553.

Navaza, J. (2001). Implementation of molecular replacement in AmoRe. *Acta Crystallogr.* D57: 1367–1372.

Ng, J.D., Gavira, J.A., García-Ruiz, J.M. (2003). Protein crystallization by capillary counterdiffusion for applied crystallographic structure determination. *J. Struct. Biol.* 142: 218-231.

Nield, J., Morris, E.P., Bibby, T.S., Barber, J. (2003). Structural analysis of the photosystem I supercomplex of cynaobacteria induced by iron deficiency. *Biochemistry* 42: 3180–3188.

Nöll, G., Kozma, E., Grandori, R., Carey, J., Schödl, T., Hauska, G., Daub, J. (2006). Spectroelectrochemical investigation of a flavoproteinwith a flavin-modified gold electrode. *Langmuir* 22: 2378–2383.

Pandit, S.B., Bhadra, R., Gowri, V.S., Balaji, S., Anand, B., Srinivasan, N. (2004). SUPFAM: A database of sequence superfamilies of protein domains. *BMC Bioinformatics* 5: 28.

Patridge, E.V., Ferry, J.G. (2006). WrbA from *Escherichia coli* and *Archaeoglobus fulgidus* is an NAD(P)H:quinone oxidoreductase. *J. Bacteriol.* 188: 3498–3506.

Peelen, S., Wijmenga, S.S., Erbel, P.J.A., Robson, R.L., Eady, R.R., Vervoort, J. (1996). Possible role of a short extra loop of the long-chain flavodoxin from *Azotobacter chrooccum* in electron transfer to nitrogenase: complete 1H, 15nb and 13C backbone assignments and secondary structure of the flavodoxin. *Biomol. NMR* 7: 315–330.

Rao, S.T., Shaffie, F., Yu, C., Satyshur, K.A., Stockman, B.J., Markley, J.L., Sundarlingam, M. (1992). Structure of the oxidized long-chain flavodoxin from Anabaena 7120 at 2 Å resolution. *Protein Sci.*1: 1413-1427.

Ross, D., Siegel, D. (2004). NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase), functions and pharmacogenetics. *Methods Enzymol.* 382: 115–144.

Simondsen, R.P., Tollin, G. (1980). Structure–function relations in flavodoxins. *Mol. Cell. Biochem.* 33: 13–24.

Stura, E.A. (1999). Seeding techniques. In: Ducruix, A., Giegé, R. (Eds.): Crystallization of nucleic acids and proteins, pp. 177 – 208. Oxford University Press, Oxford.

Talalay, P., Dinkova-Kostova, A.T. (2004). Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. *Methods Enzymol.* 382: 355–364.

Thornton, S. T., Rex, A. (1993). Modern Physics for Scientists and Engineers. Saunders College Publishing.

Tucker, D.L., Tucker, N., Conway, T. (2002). Gene expression profiling of the pH response in Escherichia coli. *J. Bacteriol.* 184: 6551–6558.

Wang, G., Alamuri, P., Maier, R.J. (2006). The diverse antioxidant systems of Helicobacter pylori. *Mol. Microbiol.* 61: 847–860.

Wolfová, J., Brynda, J., Mesters, J.R., Carey, J., Grandori, R., Kutá Smatanová, I. (2008). Crystallographic study of *Escherichia coli* flavoprotein WrbA, a new NAD(P)H-dependent quinone oxidoreductase. *Materials Structure* 15 (1): 55-57.

Wolfová, J., Grandori, R., Kozma, E., Chatterjee, N., Carey, J., Kutá Smatanová, I. (2005). Crystallization of the flavoprotein WrbA optimized by using additives and gels. *Journal of Crystal Growth* 284 (3-4): 502-505.

Wolfová, J., Kutá Smatanová, I., Brynda, J., Mesters, J.R., Lapkouski, M., Kutý, M., Natalello, A., Chatterjee, N., Chern, S.Y., Ebbel, E., Ricci, A., Grandori, R., Ettrich, R., Carey, J. (2009). Structural organization of WrbA in apo- and holo-protein crystals. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 1794: 1288-1298.

Wolfová, J., Mesters, J.R., Brynda, J., Grandori, R., Natalello, A., Carey, J., Kutá Smatanová, I. (2007). Crystallization and preliminary diffraction analysis of *E. coli* WrbA in complex with its cofactor flavin mononucleotide. *Acta Cryst.* F63: 571-575.

Yang, W., Ni, L., Somerville, R.L. (1993). A stationary-phase protein of *Escherichia coli* that affects the mode of association between the *trp* repressor protein and operator-bearing DNA. *Proc. Natl. Acad. Sci. USA* 90: 5796-5800.

Yoch, D.C. (1975). Dimerization of *Azotobacter vinelandii* flavodoxin (azotoflavin). *Arch. Biochem. Biophys.* 170: 326–333.

Paper 1

Crystallization of the flavoprotein WrbA optimized by using additives and gels

WOLFOVÁ, J., GRANDORI, R., KOZMA, E., CHATTERJEE, N., CAREY, J. AND KUTÁ SMATANOVÁ, I. .

Published in:
Journal of Crystal Growth (2005) 284 (3-4): 502-505,
doi:10.1016/j.jcrysgro.2005.07.043

Crystallization of the flavoprotein WrbA optimized by using additives and gels

Wolfová, J., Grandori, R., Kozma, E., Chatterjee, N., Carey, J. and Kutá Smatanová, I. .

Published in:
Journal of Crystal Growth (2005) 284 (3-4): 502-505,
doi:10.1016/j.jcrysgro.2005.07.043

Abstract

The tryptophan (W)-repressor binding protein A (WrbA) identified as an *Escherichia coli* stationary-phase protein was proposed as the founding member of a new family of multimeric flavodoxin-like proteins implicated in oxidativestress defense. Since WrbA is largely uncharacterized with respect to both molecular and physiological functions, the present effort is aimed at structural characterization. WrbAapoprotein was purified and used for crystallization trials at room temperature. Multicrystals of WrbA apoprotein were obtained using standard and advanced crystallization techniques. Application of additives and gelling protein for crystallization in single capillaries yielded diffraction-quality single crystals. The crystals diffracted to a resolution of 2.2Å at synchrotrons DESY (X13) in Hamburg (Germany), and Elletra (XRD1) in Trieste (Italy).

Julie Wolfová: 90% podíl na publikaci

Paper 2

Crystallization and preliminary diffraction analysis of *E. coli* WrbA in complex with its cofactor flavin mononucleotide

WOLFOVÁ, J., MESTERS, J.R., BRYNDA, J., GRANDORI, R., NATALELLO, A., CAREY, J. AND KUTÁ SMATANOVÁ, I.

Published in:
Acta Crystallographica Section F (2007) F63: 571-575
doi:10.1107/S1744309107026103

Crystallization and preliminary diffraction analysis of *E. coli* WrbA in complex with its cofactor flavin mononucleotide

Wolfová, J., Mesters, J.R., Brynda, J., Grandori, R., Natalello, A., Carey, J. and Kutá Smatanová, I.

Published in:
Acta Crystallographica Section F (2007) F63: 571-575,
doi:10.1107/S1744309107026103

Abstract

The flavoprotein WrbA from Escherichia coli is considered to be the prototype of a new family of multimeric flavodoxin-like proteins that are implicated in cell protection against oxidative stress. The present study is aimed at structural characterization of the E. coli protein with respect to its recently revealed oxidoreductase activity. Crystals of WrbA holoprotein in complex with the oxidized flavin cofactor (FMN) were obtained using standard vapour-diffusion techniques. Deep yellow tetragonal crystals obtained from differing crystallization conditions display different space groups and unit-cell parameters. X-ray crystal structures of the WrbA holoprotein have been determined to resolutions of 2.0 and 2.6 Å.

Julie Wolfová: 75% podíl na publikaci

Paper 3

Crystallographic study of *Escherichia coli* flavoprotein WrbA, a new NAD(P)H-dependent quinone oxidoreductase

WOLFOVÁ, J., BRYNDA, J., MESTERS, J.R., CAREY, J., GRANDORI, R. AND KUTÁ SMATANOVÁ, I.

Published in:
Materials Structure (2008) 15 (1): 55-57

Crystallographic study of *Escherichia coli* flavoprotein WrbA, a new NAD(P)H-dependent quinone oxidoreductase

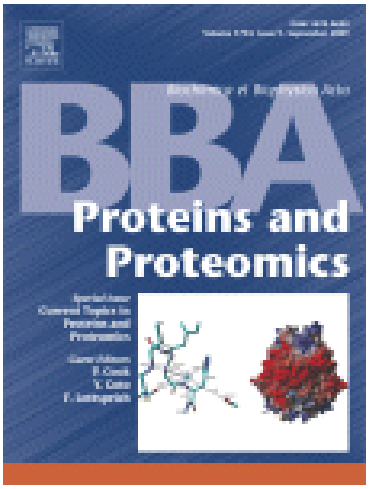
Wolfová, J., Brynda, J., Mesters, J.R., Carey, J., Grandori, R. and Kutá Smatanová, I.

Published in:
Materials Structure (2008) 15 (1): 55-57

Abstract

The flavoprotein WrbA from *Escherichia coli* represents a new family of multimeric flavodoxin-like proteins implicated in cell protection against oxidative stress. The recently revealed NAD(P)H-dependent quinone oxidoreductase activity stimulated determination of crystal structures of *E. coli* WrbA and the following search for structural features characterising the new family of redox-active proteins. Crystals obtained for *E. coli* WrbA in complex with its flavin cofactor (FMN) were subjected to X-ray diffraction analysis and the crystal structures have been determined. In order to investigate influence of FMN binding on the protein structure, WrbA apoprotein (without FMN bound) was crystallized and the diffraction data were recorded to a resolution of 1.85 Å.

Julie Wolfová: 100% podíl na publikaci



Paper 4

Structural organization of WrbA in apo- and holo-protein crystals

WOLFOVÁ, J., KUTÁ SMATANOVÁ, I., BRYNDA, J., MESTERS, J.R., LAPKOUSKI, M., KUTÝ, M., NATALELLO, A., CHATTERJEE, N., CHERN, S.Y., EBBEL, E., RICCI, A., GRANDORI, R., ETTRICH, R. AND CAREY, J.

Published in:
Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics (2009) 1794: 1288-1298, doi:10.1016/j.bbapap.2009.08.001

Structural organization of WrbA in apo- and holo-protein crystals

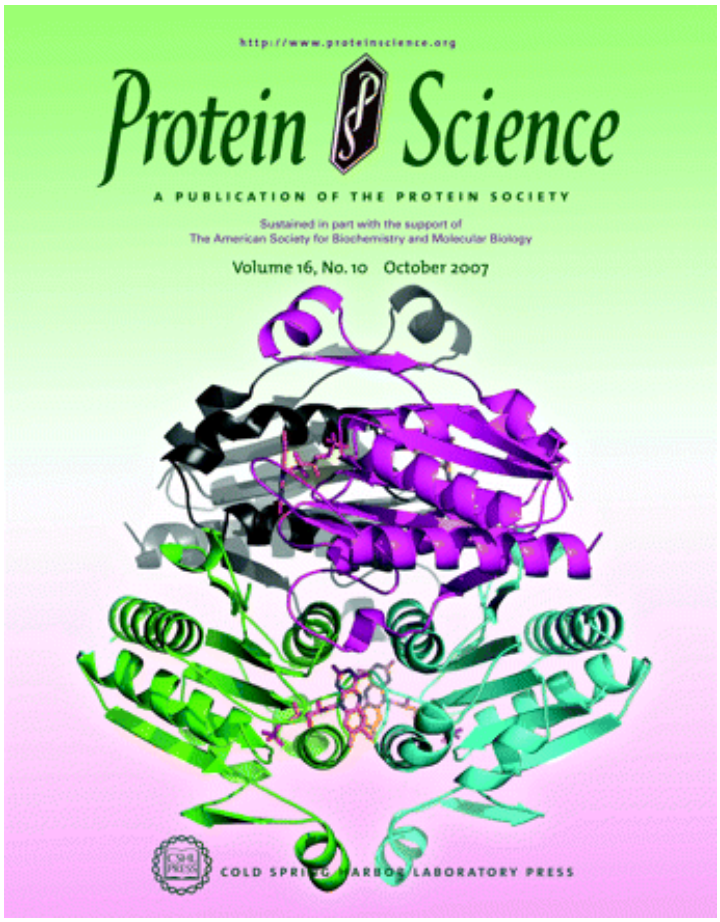
Wolfová, J., Kutá Smatanová, I., Brynda, J., Mesters, J.R., Lapkouski, M., Kutý, M., Natalello, A., Chatterjee, N., Chern, S.Y., Ebbel, E., Ricci, A., Grandori, R., Ettrich, R. and Carey, J.

Published in:
Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics (2009)
1794: 1288-1298, doi:10.1016/j.bbapap.2009.08.001

Abstract

Two previously reported holoprotein crystal forms of the flavodoxin-like *E. coli* protein WrbA, diffracting to 2.6 and 2.0 Å resolution, and new crystals of WrbA apoprotein diffracting to 1.85 Å, are refined and analysed comparatively through the lens of flavodoxin structures. The results indicate that differences between apo and holoWrbA crystal structures are manifested on many levels of protein organization as well as in the FMN-binding sites. Evaluation of the influence of crystal contacts by comparison of lattice packing reveals the protein's global response to FMN binding. Structural changes upon cofactor binding are compared with the monomeric flavodoxins. Topologically non-equivalent residues undergo remarkably similar local structural changes upon FMN binding to WrbA or to flavodoxin, despite differences in multimeric organization and residue types at the binding sites. Analysis of the three crystal structures described here, together with flavodoxin structures, rationalizes functional similarities and differences of the WrbAs relative to flavodoxins, leading to a new understanding of the defining features of WrbAs. The results suggest that WrbAs are not a remote and unusual branch of the flavodoxin family as previously thought but rather a central member with unifying structural features.

Julie Wolfová: 30% podíl na publikaci



Paper 5

WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases

CAREY, J., BRYNDA, J., WOLFOVÁ, J., GRANDORI, R., GUSTAVSSON, T., ETTRICH, R.H. AND KUTÁ SMATANOVÁ, I.

Published in:
Protein Science (2007) 16 (10): 2301-2305, doi/10.1110/ps.073018907

WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases

Carey, J., Brynda, J., Wolfová, J., Grandori, R., Gustavsson, T., Ettrich, R.H. and Kutá Smatanová, I.

Published in:
Protein Science (2007) 16 (10): 2301-2305, doi/10.1110/ps.073018907

Abstract

The crystal structure of the flavodoxin-like protein WrbA with oxidized FMN bound reveals a close relationship to mammalian NAD(P)H:quinone oxidoreductase, Nqo1. Structural comparison of WrbA, flavodoxin, and Nqo1 indicates how the twisted open-sheet fold of flavodoxins is elaborated to form multimers that extend catalytic function from one-electron transfer between protein partners using FMN to two-electron reduction of xenobiotics using FAD. The structure suggests a novel physiological role for WrbA and Nqo1.

Julie Wolfová: 10% podíl na publikaci