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Structural analysis of extrinsic proteins from the oxygen-evolving complex of photosystem II from higher plants

Ph.D. Thesis

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Institute of Systems Biology and Ecology, ASCR

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Štys, D., Schoefberger, W., Halbhuber, Z., Ristvejová, J., Mueller, N., Ettrich, R. (2005) Secondary structure estimation of recombinant PsbH, encoding a photosynthetic membrane protein of cyanobaterium *Synechocystis sp.PCC 6803*, *Photosynthetica* 43(3), 421-424.

Ristvejová, J., Kopecký Jr., V., Sovová, Ž., Balsera, M., Arellano, J.B., Green, M., Ettrich, R. (2006) Structure and dynamics of the N-terminal loop of PsbQ from photosystem II of *Spinacea oleracea*, *Biochem. Biophys. Res. Comm*. 345, 287-291.

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I hereby declare, as senior-and corresponding author of the above mentioned papers, that Jaroslava Kohoutová, in the collaboration with the co-authors, was responsible for the majority of the work, which involved molecular biology, biochemistry and experiments for structural analysis of extrinsic proteins from the oxygen-evolving complex of the photosystem II from higher plants. She contributed sidnificantly to the completion of data and the writing up of the manuscripts.

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Anotation

All life on earth depends mainly on the presence of oxygen. Largest producers of oxygen are green plants, cyanobacteria and algae. Oxygen is released from the oxygen-evolving complex of photosystem II during photosynthesis and it is used in cellular respiration of all life complexes. The oxygen-evolving complex of photosystem II has the same function in each photosynthetic organism, but it has a different composition and organization of extrinsic proteins; only PsbO protein is ubiquitous in all known oxyphototrophs.

Until now only low resolution electron microscopy structural models of plant PSII and crystal structures of cyanobacterial PSII are available. Higher plant extrinsic proteins (PsbP, PsbQ and PsbR) are structurally unrelated, non-homologues to the cyanobacterial extrinsic proteins (PsbO, PsbU and PsbV) and this is the reason why it is not possible to predict arrangement of these proteins on the lumenal site of higher plant PSII. Recently, models differ mainly in the structure of the oxygen-evolving complex, which could be resolved by determination of the exact binding sites for extrinsic proteins. An other question evolves: if the difference in the oxygen-evolving complex composition is the result of evolution or adaptation of photosynthetic organisms to their environment.

Structural knowledge of extrinsic proteins that could help to resolve the location and subsequently the function of extrinsic proteins is still incomplete. From this case, structural analysis, interactions and probably arrangement of proteins PsbP and PsbQ was studied and is described in detail in this thesis.

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1 Introduction

1.1 Photosynthesis

Photosynthesis is a process in which electromagnetic energy is converted to chemical energy used for biosynthesis of organic cell materials; a photosynthetic *organism* is one in which a major fraction of the energy required for cellular syntheses is supplied by light. Knowlegde about the existence of a photosynthetic *organism* is important because of the recent discovery of a number of aerobic bacterial species, which contain bacteriochlorophyll and carotenoids, but which are incapable of using light as the sole or major source of energy for growth [Gest, 2002]. In oxygenic photosynthesis sunlight is converted into chemical energy. This takes place in the thylakoid membrane of green plants, algae and cyanobacteria, in which the photosystem II (PS II) complex performs light-driven oxidation of water, with reduction of the plastoquinone pool and releases of molecular oxygen [Barber, 2003]. It seems that the first photosynthetic organisms able to oxidise water and evolve oxygen were cyanobacterial-like and date back about 2.5 billion years. They probably became quite widely spread throughout the sunlit surface waters of the Earth. At first, they produced oxygen that was used up in oxidising the abundant Fe^{2+} and S^{2-} in the oceans and the lithosphere. Once this great 'rust event' was completed about 2.2 billion years ago, the level of oxygen in the atmosphere rapidly grew to its modern level [Barber, 2004].

The process of photosynthesis cannot be understood without detailed knowledge of the structure of its single components. PSII is the heart of the photosynthetic process. This multisubunit complex is embedded in the thylakoid membrane of plants, algae and cyanobacteria [Barber, 2002]. The function of the PSII in different organism is identical, while the contains of subunits are different (Fig.1). From evolutionary studies of the oxygen-evolving complex it is obviously that only the extrinsic protein PsbO is ubiquitous in all oxyphytotrophs [De Las Rivas *et al*., 2004; Tohri *et al.,* 2002].

Recently the 3D X-ray structure of cyanobacterial PSII, which consist of extrinsic proteins PsbO, PsbU and PsbV was determined. It has notably improved former partial structures of the bacterial complex [Zouni *et al.,* 2001; Kamiya & Shen, 2003; Loll *et al.,* 2005; Guskov *et al.,* 2009]. Until now only low resolution electron microscopy structural models are avalaible for plant PSII [Hankamer *et al.*, 2001; Bumba & Vacha,

2003]. Cyanobacterial PSII structures provide no clues on the possible arrangement of the extrinsic proteins in the lumenal side of higher plant PSII, as extrinsic proteins PsbP and PsbQ are not structural homologs of cyanobacterial proteins PsbU and PsbV.

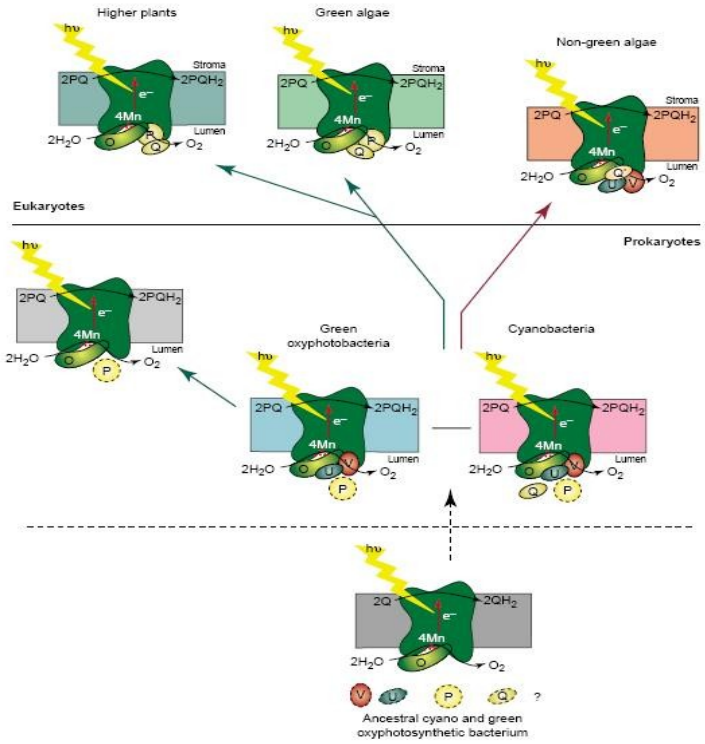


Figure 1. A simplified scheme of the distribution of oxygen evolving centre (OEC) extrinsic proteins of PSII in different types of oxyphototrophs. The extrinsic proteins that are known to be associated with the OEC are outlined with solid lines, whereas those for which there is uncertainty are outlined by broken lines and are detached from the PSII complex. The arrows in the flow diagram represent the probable evolutionary pathway from prokaryotes (cyanobacteria and green oxyphotobacteria) to eukaryotes, first to non-green algae and later, to green algae and plants. Broken lines indicate tentative relationships between extrinsic proteins and their evolutionary origin. Based on the distribution of OEC and OEC-like extrinsic proteins, is suggested that the ancestral PSII only had the PsbO extrinsic protein associated with its OEC [taken from De Las Rivas *et al*., 2004].

Detailed functional and structural studies of the photosynthetic complex from higher plants have been hampered by the impossibility to purify it to homogeneity and recently only a projection map of a newly identified particle composed by a monomeric core and antenna proteins (largest $C_2S_2M_2$ supercomplex) was obtained at 12Å resolution. This map allowed the determination of location and orientation of antenna proteins [Caffari *et al.,* 2009]. Despite the available structural knowledge and hypoteticall models (Fig.2; Fig.3) of higher plant PSII [Hankamer & Barber, 1997; De

Las Rivas *et al.,* 2007], there is not enough structural data for analysing the oxygen evolving complex (OEC) on molecular level so far.

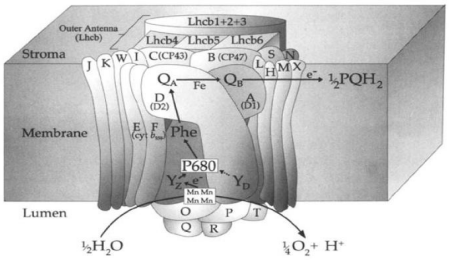


Figure 2. Model of the plant photosystem II [taken from Hankamer & Barber, 1997].

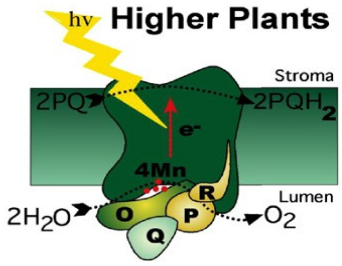


Figure 3. Model of oxygen-evolving complex of the plant photosystem II [taken from De Las Rivas *et al.,* 2007].

1.2 Oxygen evolving complex

During the process of photosynthesis oxygen (essential for life on earth) is released to the atmosphere. One of the aims of plant studies is the understanding of the mechanism and kinetics of water oxidation.

The biological function of PSII is to absorb solar light and reduce plastoquinone (PQ), producing oxygen by water oxidation according to the following reaction:

$$2H_2O + 2PQ + 4H^+_{stroma} \longrightarrow O_2 + 2PQH_2 + 4H^+_{lumen}$$

where PQ is a reversibly bound plastoquinone (QB), which forms plastohydroquinone (PQH_2) after twoelectron reduction and double protonation by protons from the stromal side of the membrane (Fig. 4). PQH_2 is displaced by another PQ from the membrane pool and released as neutral dihydroquinol, as its binding affinity for the QB cavity is relatively low. Protons from H_2O are released into the lumen, establishing the membrane pH gradient necessary for ATP synthesis [Sproviero *et al*., 2007].

The formal description of the four step reaction energetically driven by the strongly oxidizing cation radical $PSII^{+\bullet}$ with tyrosine (Y_Z) acting as redox carrier provides Kok cycle. However a deeper mechanistic understanding of oxidative water cleavage requires information on:

1. the structure of the OEC,

2. the electronic configuration and nuclear geometry of the catalytic site in each, redox state Si (S_1-S_4),
3. the reaction coordinates of the individual redox steps [Gernot, 2007].

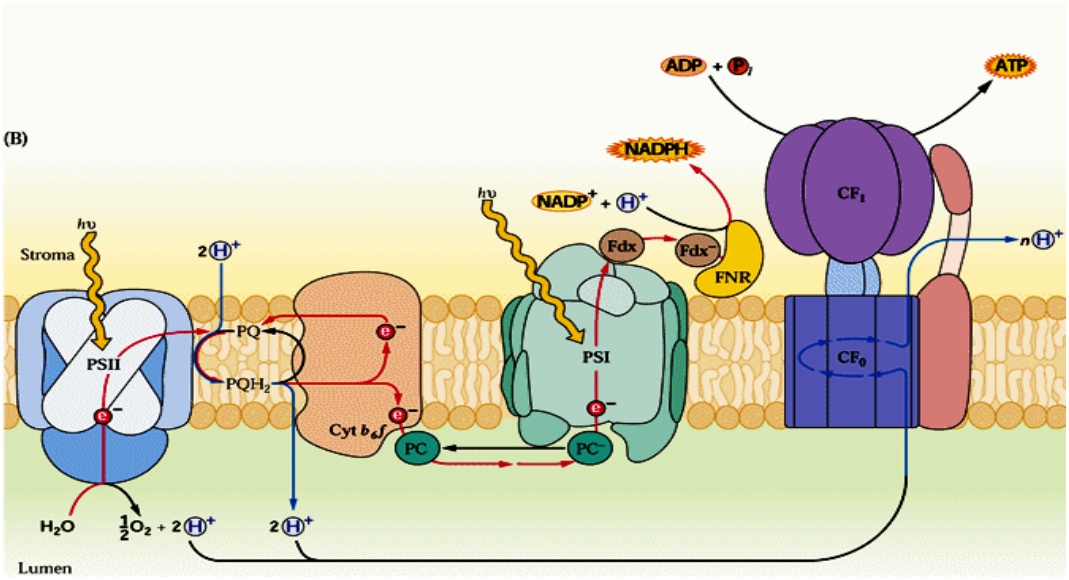


Figure 4. Model of photosynthesis [taken from Buchanan *et al.*, 2000].

The S-state cycle for the oxygen evolution reaction was first suggested by the group of Kok, modified to accommodate the model proposed by Babcock and colleagues [Barber, 2004]. The scheme so called Kok cycle (Fig.5) is based on the fact, when a photosynthetic organism is exposed to a series of saturating flashes of light, the evolution of oxygen follows a period of four. This indicates that four oxidising equivalents must be utilized at a single catalytic centre before a dioxygen molecule is formed and released. Therefore, there must be four light-induced redox turnovers of PSII and Y_Z to produce the four oxidising equivalents needed to oxidise two substrate water molecules. Dioxygen is produced on the S_4-to-S_0 transition. According to the hypothesis of Babcock and colleagues [Barber, 2004], each photo-induced step of the S state cycle (S_n-S_{n+1}) involves the removal of an electron and a proton from two bound water molecules. According to this model, electron and proton are transferred to the neutral tyrosine radical (Y_Z^*) [Barber, 2004].

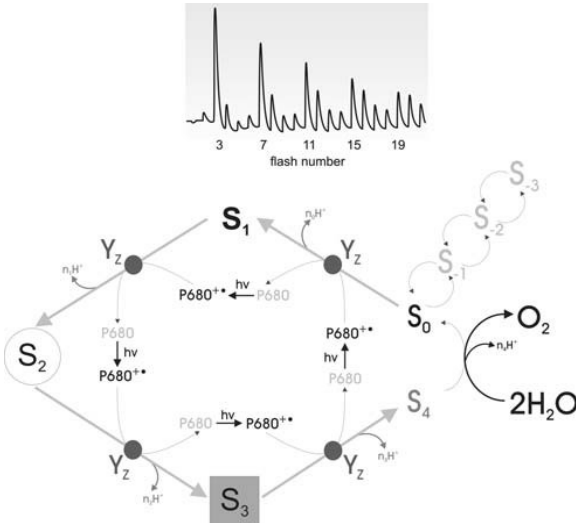


Figure 5. **Extended Kok-cycle of oxidative water cleavage.** The photooxidation of PSII+•(P680+•) is marked by arrows (hm), the intermediary redox component Y_Z is symbolized by a dark grey dot, the Si states are symbolized in the following way: the dark stable redox state S_1 by a bold capital, the metastable redox state S_2 and S_3 by capitals encircled and dark grey background, respectively, and the transient ''elusive'' state S_4 by a dashed symbol, super reduced states are marked in grey. For the sake of simplicity the slow dark relaxation reactions of S_1, S_2, and S_3 are omitted [taken from Barber, 2004].

Water splitting giving the rise to molecular oxygen, is performed on a cluster of four Mn^{2+} ions located on the lumenal side of PSII and Ca^{2+} and Cl^- ions are required for the optimal activity of this oxygen-evolving complex. The function of Ca^{2+} and Cl^- in higher plants is modulated by the presence of three extrinsic proteins named PsbO, PsbP and PsbQ and create the correct ionic environment during water oxidation that are localised at the lumenal surface [Ghanotakis *et al.,* 1984; Ghanotakis & Yocum, 1985; Miyao & Murata 1985; Seidler, 1996]. Additionally, the protein PsbR has been found in plant PSII and anticipated to play a role in water oxidation [Ljungberg *et al.,* 1986; Suorsa *et al.*, 2006].

With respect to the naming of the extrinsic proteins and oxygen evolving complex several criteria and abbreviations are used in literature. Sometimes the extrinsic proteins are named according to the mass as OEC16-16 (17) kDa protein (PsbQ), OEC23-23 (24) kDa protein (PsbP), OEC33-33 kDa protein (PsbO) and 10 kDa protein (PsbR). The PsbO protein has been refered to also as the manganese-stabilizing protein (MSP) due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants [Kuwabara *et al.,* 1989; Ljungberg *et al.,* 1986; Seidler, 1996; Zhang *et al.,* 1998; Bricker & Frankel, 2003]. In this study a naming scheme according to the genes coding for extrinsic proteins of photosystem II is used: PsbP (*psbP*), PsbQ (*psbQ*), PsbO (*psbO*) and PsbR (*psbR*). Sometimes the oxygen-evolving complex (OEC) is called by the type of its reaction: water–oxidase complex (WOC) [Seidler, 1996] and for extrinsic proteins the abbreviation OEEps (oxygen evolving extrinsic proteins) is used [De Las Rivas *et al.,* 2007]. In this thesis

we replaced these terms by commonly used terms ″extrinsic proteins″ and ″oxygen evolving complex ″.

1.2.1 The structure and function of extrinsic proteins PsbP and PsbQ

Understanding of the function of three extrinsic proteins is required to describe the oxygen evolution at physiological inorganic cofactor concentrations. There are several studies about the function of extrinsic proteins, describing as the main function of the PsbP and PsbQ proteins the modulation of Ca^{2+} and Cl^- requirements for efficient oxygen evolution and furthermore PsbO protein stabilizes the manganese cluster during exposure to low chloride concentration or to exogenous reductants [Bricker & Frankel, 2003]. The function of PsbR protein is still unknown [Suorsa *et al.,* 2006].

The function of extrinsic proteins is related to their structure. Therefore structural knowledge and models are helpful to propose the water oxidizing mechanism. This thesis contributes to the structural and functional knowledge of the proteins PsbQ and PsbP.

1.2.1.1 The structure of the PsbQ protein

The secondary structure of PsbQ was studied by FTIR spectroscopy, CD spectroscopy; using bioinformatic methods [Balsera *et al.*, 2003a] as well as by X-ray diffraction studies. The smallest extrinsic protein of oxygen-evolving complex PsbQ first extracted and purified from spinach PSII membranes was crystallized and its structure was determined by X-ray diffraction analysis to the resolution of 1.95 Å [Calderone *et al.*, 2003]. Similar results to the one reported for the native spinach PsbQ fragment were obtained for the recombinant PsbQ protein of PSII from *Spinacia oleracea* [Balsera *et al.*, 2005]. The overall shape is modelled as the result of the contribution of two different structural regions: the N-terminal region (residues 1–44) and the C-terminal region (residues 45–149). The C-terminal region is folded as a four up-down helical bundle with short loops connecting the helices (Fig. 6).

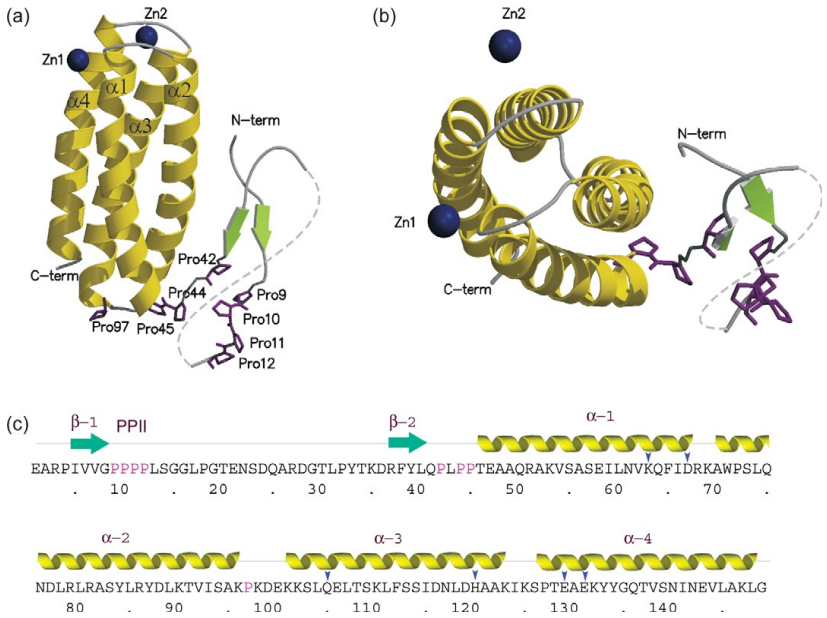


Figure 6. Overall structure of spinach PsbQ. A ribbon representation of PsbQ structure (a) side view, (b) top view. The broken line represents the unmodelled residues 14–33 in the crystal structure. Zinc ions are shown as blue spheres. Proline residues of the Pro-rich region are labelled and drawn in sticks representation. (c) Primary and secondary structure (based on DSSP) of crystallized PsbQ. Protein residues involved in Zn^{2+} coordination are labelled with blue triangles. Proline residues of the Pro-rich region are coloured magenta [taken from Balsera *et al.,* 2005].

The high–resolution structure of psbQ was obtained by X-ray diffraction analysis from data measured to the resolution of 1.49 Å [Balsera *et al.,* 2005; pdb code: 1VYK]. The N-terminal portion, that possibly interacts with the photosystem core, was not visible in the crystal of native PsbQ protein, probably because of natural flexibility [Kuwabara, 1992; Calderone *et al*., 2003].

On the contrary, the N-terminal region, the first reported for a member of the PsbQ family, is loosely packed, showing extended conformational elements and two short β-strands. Despite the high resolution (1.49 Å), the loop from residues 14–33 showed significant disorder in the crystal structure; no structure for this region could be assigned. A key motif in the N-terminal region is the polyproline type II (Fig.7) pattern formed by four Pro residues (residues 9–12) [Balsera *et al.,* 2005].

The N-terminal region contains a number of charged residues, some of these are fully conserved in the PsbQ sequence subfamily of higher plants (Glu24, Arg27, Glu28, Lys35 and Arg37). They may participate in the electrostatic interaction with the OEC extrinsic or intrinsic proteins of PSII. Furthermore, Pro residues ranging from 9 to 12 are all in the trans configuration, forming a rather exposed PPII structure.

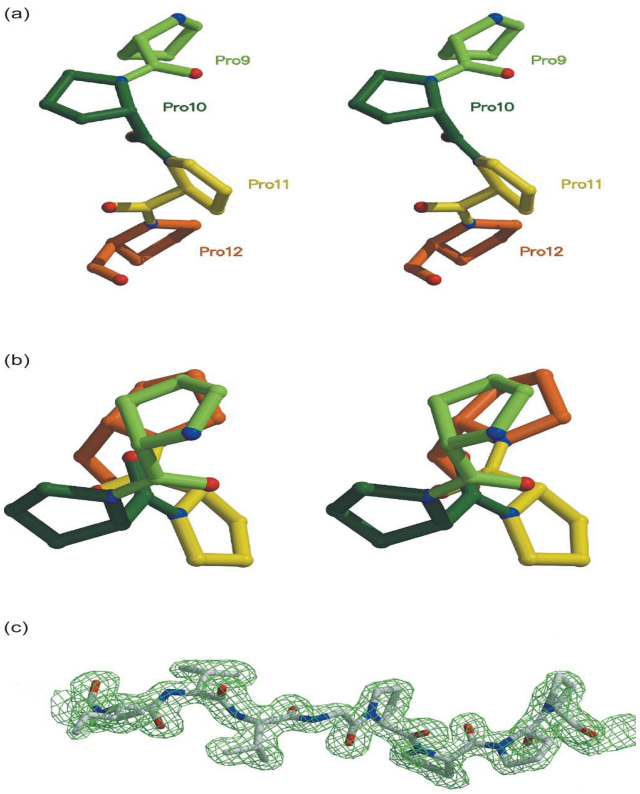


Figure 7. Polyproline type II structure in PsbQ. Stereo view of the PPII structure with each Pro residue coloured differently; (a) side view, (b) top view. The 9–12 Pro residues form a PPII structure that is characterized by having a lefthanded structure and an axis of 3-fold symmetry. (c) Electron density map in the PPII structure. A fragment of the crystal structure of the recombinant spinach PsbQ spanning residues 5–12 (Ile-Val-Val-Gly-Pro-Pro-Pro-Pro) is shown overlaid with its corresponding $2F_oKF_c$ electron density map in green at 1s [taken from Balsera *et al.,* 2005].

This structural motif is often involved in the regulation of weak protein–protein interactions and may confer flexibility to the structure, since its geometry allows to progress immediately into α-helix, β-strand, or turn. Likewise, the presence of neighbouring Gly residues is probably critical in forming structural hinges or alternative tertiary conformations [Balsera *et al.,* 2005].

1.2.1.2 The structure of the PsbP protein

The secondary structure of the extrinsic protein PsbP was first determinated by using FTIR spectroscopy. Using this technique, structural studies were performed in solution at various physiological temperatures and pHs with minimal sample preparation, which is not accessible to other techniques such as X-ray crystallography and nuclear magnetic resonance [Zhang *et al.,* 1998]. An interesting feature of PsbP structure is that this protein contains a large proportion of extended β-sheet structure (37%), but only 5% is α-helical. Mentioned structural feature is in clear contrast to the secondary structure of the reaction centre of photosystem II (PSII RCs),

which has high content of α-helical structure (40-62%) that was also determined by FTIR spectroscopy [Zhang *et al.,* 1998].

The structure of PsbP was more elucidated by X-ray diffraction analysis, even that structural details and function of PsbP domain proteins are still completely unknown [Ifuku *et al.,* 2004]. For X-ray analysis the protein PsbP from *Nicotiana tabacum* was expressed in *Escherichia coli*, subsequently purified and crystallized. It was found the nine amino-terminal residues of PsbP are easily degraded during crystallization. As these residues are functionally dispensable, they were genetically removed [Ifuku *et al.,* 2003] and the crystal structure was determined at the resolution of 1.60 Å.

The structure of PsbP lacking the N-terminal nine amino acids was determined by the multiwavelength anomalous diffraction (MAD) method with a Hg derivative. The electron densities of the N-terminal portion (residues 10–15) and two loop regions (residues 91–107 and 138–140) have not been seen in the model because of disorder. The structure of PsbP is based on central six-stranded antiparallel β-sheet covered on both sides by α-helices (Fig. 8).

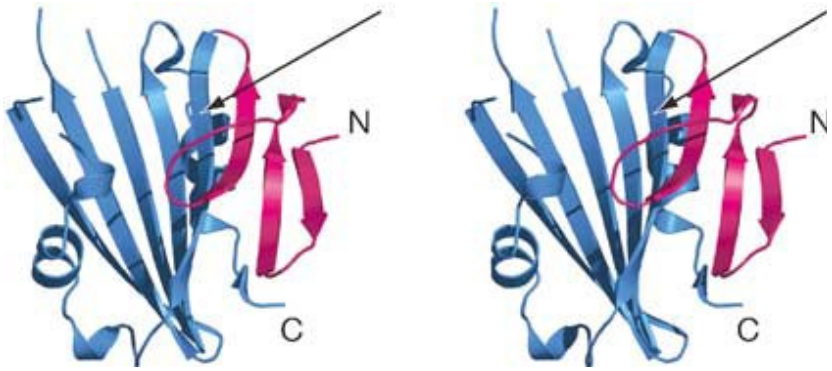


Figure 8. Stereo view of the structure of PsbP. The schematic representation of the PsbP structural model shows a series of β-strands that are derived from residues 16–53 (domain I: pink) and the central six-stranded antiparallel β-sheet flanked on both sides by helices (domain II: blue). The N (residue 16) and C (residue 186) termini are labelled. The position of the Asn 58–Val 59 bond is indicated by an arrow [taken from Ifuku *et al.,* 2004].

The segment of Thr 16–Phe 53 forms an antiparallel β-sheet and one β-strand (domain I) that back the central sheet (domain II). The existence of two distinct domains in PsbP is showing that the Asn 58–Leu 59 bond in tobacco PsbP (which corresponds to the Asn 58–Val 59 bond in spinach PsbP) becomes susceptible to proteolysis under

moderately denaturing conditions [Ifuku *et al.,* 2004]. The exchange of domain I among PsbPs from several plant species has been shown to affect significantly the ion-retention activity of these hybrid proteins, suggesting that domain I has an important role in ion-retention activity in PS II [Ifuku & Sato, 2001].

In the later study of truncated PsbP [Ifuku *et al.,* 2005a] previous biochemical work about PsbP [Ifuku & Sato, 2002] in terms of its 3D structure was reconsidered, and it was confirmed that the N–terminal flexible region of PsbP, which cannot be seen in the crystal structure, is essential for Ca^{2+} and Cl^- retention in PSII. Now exists the hypothesis that for ion-retention activity of PsbP of higher plants conserved residues especially positively charged are necessary. PsbP protein has highly conserved residues of Gly-10, Lys-11 and Lys-13 in the N-terminal. Recently, it is suggested that the positively charged lysyl residues on PsbP would be involved in electrostatic interaction with other PSII subunits [Tohri *et al.,* 2004]. In fact, an introduction of negatively charged residues into N-terminal region of PsbP was shown to lower the ion-retention ability of PsbP [Ifuku & Sato, 2001].

1.2.1.3 The function of extrinsic proteins PsbP and PsbQ

The general function of extrinsic proteins PsbQ and PsbP is in stabilizing the manganese cluster. There are some hypothesis describing the detailed functions of extrinsic proteins PsbP and PsbQ. It is known that the PsbP protein plays a key role in the stabilization of the Mn cluster, once finally assembled [Kavelaki & Ghanotakis, 1991] and this protein has an additional important function in delivering Mn to PSII as well as during the assembly of the Mn cluster: On the one hand the PsbP protein is a Mn storage protein [Bondarava *et al.,* 2005]. On the other hand exits high evidence that Mn-binding to PSII is necessary for a tight binding of the PsbP protein. However, its absence is not lethal but slows down the process of photoactivation.

The PsbQ protein has been observed to increase the binding affinity of Cl^- to PSII (maintaining an optimal concentration of Cl^- in PSII) and the PsbP protein to be involved in increasing the binding affinities of both Cl^- and Ca^{2+} [Ghanotakis *et al.,* 1984a,b; Miyao & Murata 1984a,b; Bondarava *et al*., 2005]. Experiments with mutants of *Nicotiana tabacum* lacking proteins PsbP, PsbQ showed that the lack of PsbP

severely impaired the photochemical reaction of PSII in the light and led to disassembly of the Mn cluster in the dark, whereas the lack of PsbQ did not alter the plant phenotype. The PsbP is indispensable for plant survival and normal PSII functions in higher plants *in vivo*, while PsbQ is not [Ifuku *et al*., 2005b].

Other functions proposed for PsbP and PsbQ are:

1. to form a gate that is open for substrates and products, but closed to nonphysiological reducing agents [Hillier *et al*., 2001; Anderson, 2001; Van der Meulen *et al*., 2002],
2. to create a low dielectric medium optimal for PSII binding to Ca^{2+} and Cl^{-} [Vrettos *et al*., 2001; Wincencjusz *et al*., 1999],
3. to tune up the magnetic properties of the Mn cluster [Campbell *et al*., 1998].

In addition, PsbQ can also play a role in maintaining the overall stability of PSII. PsbQ has been reported to be thermostable, with a melting point of ~ 65°C. This is compatible with some of features found in the PsbQ sequence. PsbQ favors Arg (5.4%), but avoids the thermolabile Cys and His in all its sequence and Pro (except Pro72) in its four α-helices. The respective frequency of these residues is related to the thermostability of proteins, suggesting that PsbQ could fulfill these prerequisites. Moreover, salt bridges formed between residues that are relatively close to each other in the sequence are also known to stabilize proteins. Particularly, PsbQ has several Arg and Lys residues sequentially close to Glu and Asp residues, which could form salt bridges (i.e. Arg27 and Glu28, Glu36 and Arg37, Glu47 and Arg51, Glu67 and Arg68, Lys102 and Glu106, Asp100 and Lys101, Asp130 and Lys131). This, favours the suggestion that PsbQ is a thermostable protein. PsbP has also been suggested to be thermostable. All in all, PsbQ in conjunction with PsbP could play a functional role in keeping Ca^{2+} and Cl^{-} bound to the oxygen-evolving complex, as well as a structural role in maintaining the overall (thermo)stability of PSII [Balsera *et al*., 2003b].

Recently, the functional role and properties of poorly characterized PsbR protein are studied. It has been proposed that PsbR is located on the lumenal side of PSII and is involved in binding of extrinsic proteins, although no direct *in vivo* experimental proof is available [Ljungberg *et al*., 1986]. Experiments show, that the absence of PsbR clearly diminishes the oxygen evolution capacity of thylakoid membranes, indicating that PsbR is essential for optimization of photosynthetic water splitting and electron transfer in PSII. Also it was shown that the lack of PsbR induced a reduction in the

content of both PsbP and PsbQ proteins, and a near depletion of these proteins was observed under steady state low light conditions. This regulation occurred post-transcriptionally and likely involved a proteolytic degradation of PsbP and PsbQ proteins in the absence of an assembly partner, proposed to be the PsbR protein. Stable assembly of PsbR in the PSII core complex was shown to require a chloroplast-encoded intrinsic low molecular mass PSII subunit PsbJ. According to this hypothesis the PsbR protein is an important link in the PSII core complex for the stable assembly of the oxygen-evolving complex protein PsbP, whereas the effects on the assembly of PsbQ are probably indirect. The physiological role of PsbR, PsbP and PsbQ proteins is discussed in the light of their peculiar expression in response to growth light conditions [Suorsa *et al*., 2006].

Quite conversely, it was recently speculated that, in addition to the role of extrinsic protein in water splitting and oxygen evolution, higher plant OEC proteins might also have structural roles; PsbP and PsbQ are possibly important for the stacking of grana thylakoids, which is known to become more pronounced upon acclimation of plants to low light. In line with this suggestion, PSII complexes in non-grana bundle sheet chloroplasts of maize and *Sorghum* are depleted in PsbR, PsbP, and PsbQ proteins as compared with mesophyll chloroplasts with differentiated grana and stroma thylakoids. From different light treatments of A*rabidopsis* WT and *psbR* plants, it is clear that the stoichiometry of the PsbR, PsbP, and PsbQ proteins with respect to the PSII core and the PsbO protein can be highly variable, and novel functions for these proteins are likely to be discovered [Suorsa *et al*., 2006].

De las Rivas and colleagues (2007) mention the proposed hypothesis for a possible function of extrinsic proteins in the correct 3D assembly of the grana lamellae needed for higher plant PSII full activity and regulation; including a particular role for PsbQ in protein–protein interactions needed for such assembly. This is a new hypothesis presented as an integrative approach to better understand the dynamic function of PSII in higher plant thylakoids. A model for this hypothesis (Fig. 9) suggest some relationship between the higher plant "PSII extrinsic proteins" and the higher plant "PSII functional state" [De Las Rivas *et al*., 2007]. In this way, oxygen-evolving assembly, grana stacking, light harvesting complexes (LHC) plugging, PSII linear electron transfer (ET), and PSII reaction centers opening will occur only when extrinsic proteins are fully attached to the PSII. In fact, PsbP and PsbQ have been found to

associate with PSII only in the grana thylakoids [De Las Rivas *et al*., 2007; Hashimoto *et al*., 1997]. By contrast, oxygen evolving disassembly, grana destacking, LHC unplugging, PSII linear ET interruption, non-photochemical quenching (NPQ) increase, and PSII reaction centers closing are conditions in that PsbQ, PsbP, PsbR, and maybe PsbO will be most probably detached from PSII.

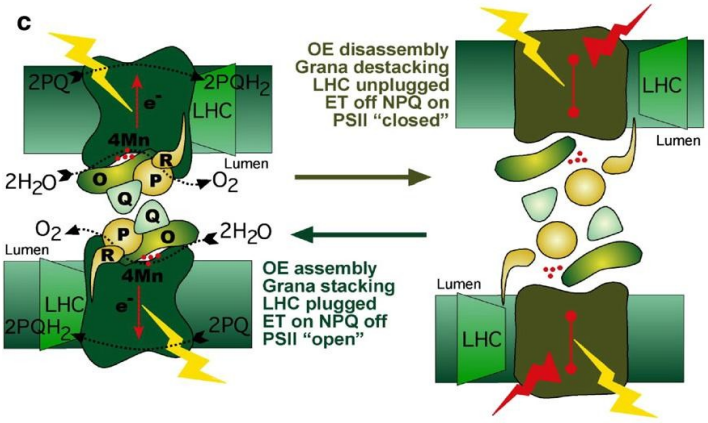


Figure 9. Schematic drawing showing a proposed role for extrinsic proteins in the correct 3D assembly of the grana lamellae and in PSII full activity [taken from De Las Rivas *et al*., 2007].

1.2.2 Interactions of extrinsic proteins PsbP and PsbQ

To study the assembly of extrinsic proteins in the oxygen-evolving complex, structural knowledge is used. As it was recently reported, the treatment of PSII enriched samples (so called BBY fractions) of spinach with mercury resulted in a release of PsbO, whereas both PsbP and PsbQ still remained bound to PSII. This finding corroborates with results obtained with spinach PSII submembrane fractions exposed to Hg and resulting in a 40% depletion of PsbO, but maintaining the normal association of PsbP and PsbQ in PSII. Taking into a consideration the possibility that mercury treatment has caused an artificial binding of PsbP and PsbQ to intrinsic subunits of PSII, these results are still contradicted with the ‘‘regulatory gap’’ model proposing that PsbP associates to PSII solely via the PsbO protein, and rather supports the concept that either all extrinsic proteins are independently bound to PSII. Only PsbO and PsbP are independently bound to PSII and one or both of them provide a docking site for PsbQ [Nield *et al.,* 2002; Bricker & Frankel, 2003; Meades *et al.,* 2005; Suorsa & Aro, 2007].

Bricker and Frankel (2003) suggested that the maintenance of PsbP and PsbQ in PSII after the removal of PsbO do not necessarily exclude the importance of PsbO in binding of the PsbP protein. After PsbP is stably associated with PSII, conformational changes can take place in intrinsic subunits of PSII and the interaction of these proteins can keep PsbP in the PSII complex despite the removal of the principal assembly partner, PsbO. In line with this suggestion, the importance of PsbR particularly for the assembly of PsbP was recently demonstrated [Suorsa *et al.,* 2006]. It should be emphasized that the *psbR* mutant contained PsbO in amount comparable to wild-type plants. This finding provides strong support for the model that the association of PsbP takes place independently of PsbO, the primary assembly partners likely being the intrinsic PSII protein(s) PsbR (and PsbJ).

PsbQ was found to be completely missing from a tobacco mutant lacking the PsbP protein, and having a decreased amount of the PsbO protein [Ifuku *et al.,* 2005b]. However, the PsbP-less mutant contained a slightly smaller protein fragment of PsbQ, which was suggested to be a degradation fragment of the PsbQ protein, not able to assemble due to the lack of the proposed assembly partner, PsbP [Ifuku *et al*., 2005b]. On the other hand, PsbO probably contributes to the association of PsbQ, deduced from the finding that despite nearly normal presence of PsbP. PsbQ was practically missing in an *Arabidopsis thaliana* mutant having only low amounts of PsbO [Yi *et al*., 2005]. Recently it was reported that although the lack of PsbR resulted in decreased contents of both PsbP and PsbQ. The amount of PsbQ was, however, remarkably higher than the amount of PsbP [Suorsa *et al*., 2006]. The normal presence of PsbO in the *psbR* mutant can allow partial association of PsbQ despite the stable assembly of PsbP being hindered. In line with these studies, structural analyses of extrinsic proteins proposed that PsbQ and PsbP interact with different parts of PsbO. Moreover, the negatively charged area of PsbO complements with the positive charged area of the PsbQ protein, so it was suggested that PsbQ docks itself to PsbO through an electrostatic force [Suorsa & Aro, 2007].

Recently there were some modeling attempts speculating about the location and arrangement of extrinsic proteins (PsbP, PsbQ, PsbR associated with PsbO) in PSII from plants and algae [Hasler *et al.,* 1997; Hankamer et al., 2001; De Las Rivas *et al.,* 2007]. Results from the study of De Las Rivas (2007) are combined with konwledge from literature. The hypothesis (Fig. 10a) shows a drawing depicting a schematic

arrangement of PsbO, PsbP, PsbQ and PsbR within PSII extrinsic region. PsbO, in algae and plants, is located closest to the oxygen-evolving Mn catalytic center with an elongated shape coming towards the lumen [De Las Rivas *et al.,* 2007]. This is based on structural analysis using PSII diffraction data, considering PsbO from plants to be very similar to cyanobacterial PsbO and conserved along evolution.

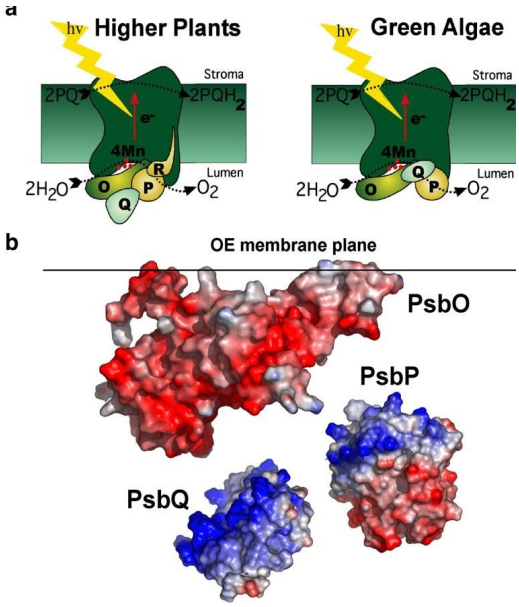


Figure 10. Models designed by bioinformatic methods a) Schematic drawing of PSII showing the proposed arrangement of PsbO, PsbP, PsbQ and PsbR in the extrinsic lumenal region. b) 3D surface models of PsbO, PsbP and PsbQ, built using the PDBs [De Las Rivas *et al.,* 2007].

However, the figures (Fig. 10) propose two main differences between green algae and higher plants:

1. a closer interaction between PsbQ and PsbP in plants,
2. a larger external interacting N-terminal domain present only in PsbQ from plants.

The model was compared with the structural analysis of the diffraction data getting from crystals of PsbO, PsbP and PsbQ and a low resolution model of higher plant PSII derived from electron microscopy, that also indicated that PsbP interaction interface is most probably closer to the head domain of PsbO and PsbQ interaction interface is closer to the cylinder external domain of PsbO (Fig. 10b).

1.2.3 Attachment of extrinsic proteins and their role in grana stacking

Nowadays at least three models have been suggested for the mutual order of assembly of higher plant extrinsic proteins [Suorsa & Aro, 2007]. Perhaps the most

widely accepted model proposes that from extrinsic proteins, PsbO is the only one primarily binding to the lumenal side of PSII, and thereby provides a docking site for PsbP, which, in turn, associates the PsbQ protein (Fig. 11). This hierarchical model, also referred as the ‘‘regulatory cap’’ model, is originally based on biochemical reconstitution studies, and has also been supported by the results obtained from electron cryomicroscopy analysis of isolated spinach PSII-LHCII supercomplexes. Moreover, the characterization of the carboxylate groups of the PsbO protein revealed certain residues that are needed for the proper binding of the PsbP protein. In higher plants, alternative models for the ‘‘regulatory cap’’ model are emerging for the assembly of extrinsic proteins.

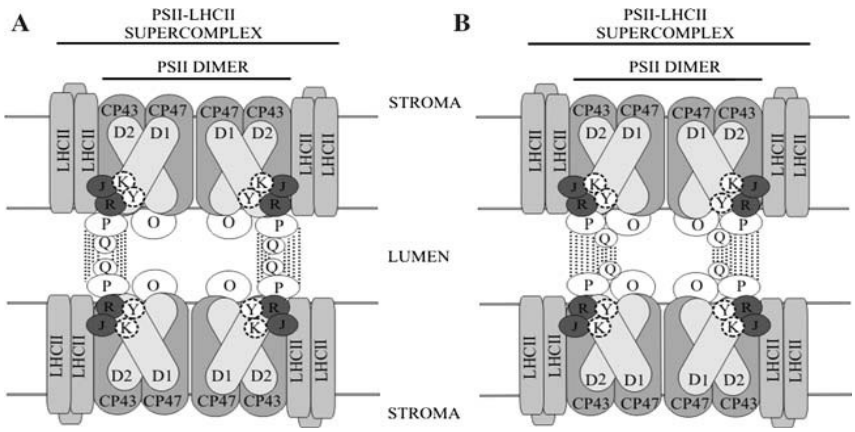


Figure 11. Two hypothetical schemes depicting the binding of the extrinsic proteins to the PSII core in higher plant chloroplasts. It is not known whether (A) PsbQ is attached only to PsbP or (B) whether it needs both PsbO and PsbP for stable attachment. The role of PSII lowmolecular- mass proteins PsbR and PsbJ for the binding of PsbP is emphasized. It still remains unclear whether PsbR (and PsbJ) directly binds PsbP, or whether it (they) only fine-tune the structure of the PSII lumenal side, thus making the association of PsbP more feasible. PsbK and PsbY possibly have a role in the assembly of PsbO (and the other extrinsic proteins). Single letters represent the respective Psb proteins. Note that the PsbJ, PsbK and PsbY proteins in higher plants are membrane spanning and the exact location of the proteins is not yet known. The role of PsbP and PsbQ in lumenal interactions, which possibly also affect grana stacking, is depicted as dash lines [taken from Suorsa & Aro, 2007].

According the results obtaining from study of supercomplexes by Kirchhoff and colleagues (2007), another model is avalaible (Fig.12). This model focuses on the PSII–LHCII supercomplex organization (probably PSIIα centers).

In the mean supercomplex PSII are connected with 4 LHCII-trimers under stacked conditions and are excitonically coupled (connectivity) and well separated from PSI in unstacked regions (Fig.12-upper part). Unstacking leads to randomization of the

protein arrangement and the separation of LHCII-trimers from PSII. PSI and PSII come in excitonic contact (spillover). Data indicate that the low-salt treatment does not result in a structural monomerization of PSII-dimers. The functional monomerization, i.e. whether the dimers are excitonically uncoupled under these conditions, is still unclear as symbolized by the question mark (Fig.12-middle part). The white circles at the PSII supercomplexes indicate unoccupied binding sites for LHCII-trimers. Restacking of unstacked thylakoids leads almost to the same macromolecular organization as in untreated membranes, i.e. rebinding of LHCII-trimers to PSII and a resagregation of PSII and PSI (Fig.12-lower part). It is proposed a redistribution of minor LHCIIs from PSIIβ centers to PSIIα, where they can attach to one of the LHCII-trimer binding sites (exclamation point). This can result in a decreased connectivity between PSIIα centers [Kirchhoff *et al.,* 2007].

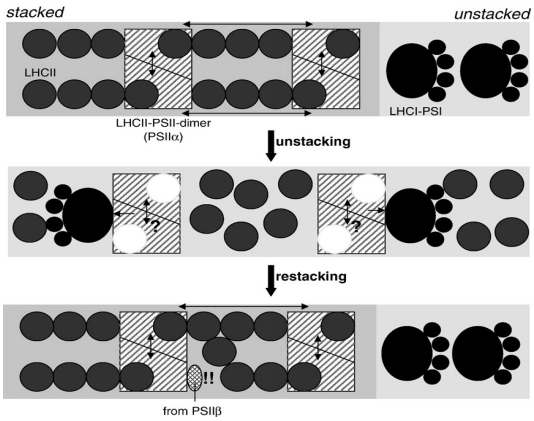


Figure 12. **Model illustrating the changes in the protein organization upon unstacking and restacking of thylakoid membranes** [taken from Kirchhoff *et al.*, 2007].

Questionable are interactions of extrinsic proteins in grana stacking coming from this model. It seems that location of potential binding areas of the extrinsic proteins could resolve not only the structure of the oxygen-evolving complex and the mechanism of oxygen evolving reaction, but also it could be used to resolve the question of grana stacking.

2 Materials and methods

Materials and methods are described in respective publications. In this chapter additional information with respect to the modified PsbQ purification protocol used for NMR measurements and crosslinking reactions for interaction studies is given.

2.1 Purification of the recombinant PsbQ protein

The cells *Escherichia coli* transformed by vector JR2592 (B96 cells) [Balsera *et al.,* 2003a] were grown at 37°C in M9 media [Sambrook *et al.,* 2001] supplemented with trace elements [Neidhart *et al.*, 1974] and thiamine; in double labelled M9 media supplemented with BioExpress solution [Rhima *et al*., CIL application] and 100 µg/ ml ampicillin and 20 µg/ml chloramphenicol [Sambrook *et al.,* 2001]. When the optical density of the culture reached the value of 0.6 at 550 nm, the expression of the labelled recombinant PsbQ was initiated by adding 1 mM IPTG. After 15-18 hours incubation at 30 °C, the cells were harvested by centrifugation, suspended in 0.8 M Tris-HCl pH 8.1, containing 1 mM phenylmethanesulfonyl fluoride (PMSF), and then French-pressed. PMSF was present in all subsequent buffers. Unbroken cells were removed by centrifugation at 7 000x*g* for 15 min. The supernatant was collected and precipitated with 100% $(NH_4)_2SO_4$. The precipitant was suspended in 20 mM Tris-HCl pH 8.0, 1 mM EDTA (buffer A) and dialyzed against buffer A at 4 °C overnight. Unsolubilized material was removed by centrifugation at 15 000x*g* for 45 min. The protein solution was passed through inonex-exchange columns (DEAE column connected with SP column; GE Healthcare Bio-Sciences AB Uppsala, Sweden) preequilibrated with buffer A. The PsbQ protein was eluted from the SP column using a salt linear gradient up to 0.4 M NaCl for 20 min. at a flow rate of 2 ml/min. To test the purity of sample the SDS polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli *et al.,* 1970] with a total acrylamide content of 12% in the separating gel was used. The sample was concentrated using centrifuge filter devices (Amicon Ultra 10.000 MWCO, 15 ml capacity; Millipore (Billerica, MA)) up to concetration of 3.5 mg/ml. This sample was used for NMR measurement.

2.2 Crosslinking reactions

A. Crosslinking reagents (solvent):

DSS (DMSO 10%)

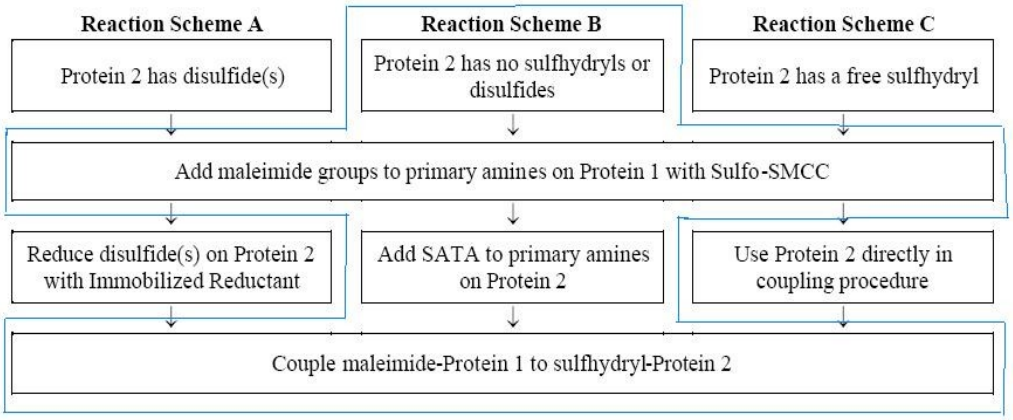
DSG (DMSO 10%)

DST (DMSO 10%)

EDC (dH_2O)

Recombinant proteins HisPsbP, PsbP and PsbQ were washed by buffers recommended for each crosslinking reagent [manual of manufacturer 62-65, Pierce] and concetrated using centrifuge filter devices (Amicon Ultra 10.000 MWCO, 4 ml capacity; Millipore (Billerica, MA)) up to concetration of 1mg/ml; 2mg/ml and 5 mg/ml. The crosslinking reactions were performed at both 4°C and 21°C in recommended buffers [manual of manufacturer 62-65, Pierce]. Several possibilities according to the recommendation of the manufacturer of crosslinking reagents have been tried. The mixture of proteins and crosslinking reagents was incubated for 2 hours at 21°C and overnight at 4°C. Samples were washed by buffers using centrifuge filter devices (Amicon Ultra 10.000 MWCO, 4 ml capacity; Millipore (Billerica, MA)) to get out not bind crosslinking reagents. The reactions were analysed by SDS-PAGE with a total acrylamide content of 12% in the separating gel [Laemmli *et al.,* 1970].

B. *Two step crosslinking reaction*:



Scheme 1. Controlled protein-protein crosslinking reaction [taken from manual of manufacturer 61, Pierce].

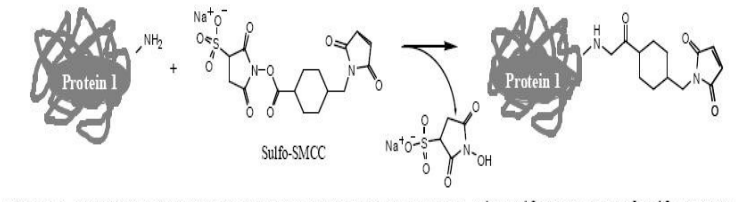
Crosslinking reagents (solvent):

SulfoSMCC (phosphate buffer saline)

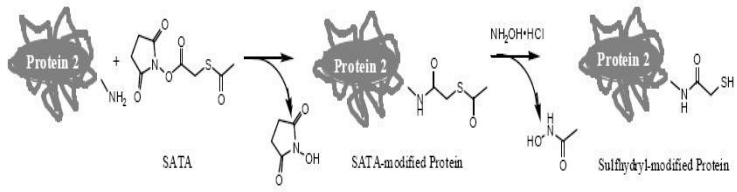
SATA (DMF)

Recombinant proteins HisPsbP, PsbP and PsbQ were washed by recommended phosphate buffer saline and concetrated using centrifuge filter devices (Amicon Ultra 10.000 MWCO, 4 ml capacity; Millipore (Billerica, MA)) up to the concetration of 5 mg/ml. The crosslinking reaction with slight modifications was performed according manual of manufacturer (Scheme 1, Fig.13) [manual of manufacturer 61, Pierce].

a) Maleimide Activation of Protein 1



b) Sulfhydryl Modification of Protein 2



c) Crosslinking reaction of the activated Protein 1 and Protein 2

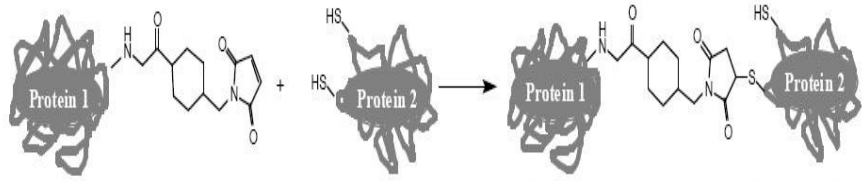


Figure 13. Chemical crosslinking. a) The NHS ester of SATA reacts with primary amines on lysine residues. Hydroxylamine de-protects the latent sulfhydryl groups, which are able to react with maleimides. b)The Sulfo NHS ester of Sulfo – SMCC reacts with primary amines on lysine residues resulting in the protein containing an available sulfhydryl - reactive maleimid group. c) The double bond of the maleimid reacts with sulfhydryl groups at pH 6,5 – 7,5 to form a stable thioether bond [taken from manual of manufacturer 61, Pierce].

The cleaning of samples from unbound crosslinking reagents was performed by using centrifuge filter devices (Amicon Ultra 10.000 MWCO, 4 ml capacity; Millipore

(Billerica, MA)). Reactions were analysed using SDS-PAGE with a total acrylamide content of 12% in the separating gel [Laemmli *et al.,* 1970] as well as using Western blotting [Cutler, 2004] with antibodies anti-PsbP and anti-PsbQ (AntiProt, Germany).

The protocol was used to study interactions of PsbP/Cys159Ala with PsbQ and PsbP/Met19Ala with PsbQ. Mutants of PsbP protein were prepared by method Overlap extension site direct mutagenesis [Sambrook *et al.,* 2001] and analysed by commercial sequencing.

3 Discussion and summary

In our research, we used a step by step approach to gain structural and functional insight into the OEC. To understand the behaviour of the mentioned proteins, firstly the single proteins were characterized on an atomic scale, and later their interactions and possible complexes were studied.

3.1 Structural analysis of the PsbQ protein

The recently determined structure of PsbQ protein by X-ray crystallography at the resolution of 1.49 Å [Balsera *et al*., 2005] lacks the N-terminal loop region probably due to its native flexibility, despite the generally high resolution of the structure. However, the N-terminal region of PsbQ protein contains a number of charged residues, some of them are fully conserved in the PsbQ sequence subfamily of higher plants, and therefore may participate in the electrostatic interaction with the OEC extrinsic or intrinsic proteins of PSII [Balsera *et al.*, 2005]. Knowledge of the structure of this loop region and especially knowledge of its flexibility is a key issue in assesing the interaction of this protein with PSII or other OEC proteins. Recombinant expression and purification of the PsbQ protein stable in high concentrations enabled us to analyze its secondary structure by Raman and FTIR spectroscopy and compare the results with our structural model by restraint-based homology modeling [Ristvejová *et al*., 2006]. Additionally, molecular dynamics simulations in explicit solvent at 300K enabled us to examine the flexibility of the loop region and the protein as a whole. Simulations with a construct lacking the first 12 amino acids can be directly correlated to experimental results and explain the non-recognizing of PsbQ by PSII of this construct [Ristvejová *et al*., 2006]. Asp24 together with Lys101 and Lys102was identified to play a potential key role in the interaction with either PsbP or PSII, in general. Our proposed model makes very clear suggestions for the structure of the Polyproline II motif in the N-terminal loop region, demonstrating its stablizing role in the flexibility of the loop by anchoring the loop region approximately in the middle around residue 25. Together with Pro42, Pro44, and Pro45 the polyproline type II pattern forms a gate for the

returning loop that does not show any hydrogen bonding with the prolines and that is held in position by steric hindrance only.

To resolve the Polyproline II motif and its influence on the flexibility of the N-terminal loop region experimentally, NMR spectroscopy was applied. Our recombinant PsbQ construct was used for analysis by NMR spectroscopy with fullfilling additional conditions as stability in phosphate buffer for two weeks at 4 °C in a concentration range of 200 μM – 500 μM (3,4 mg/ml-8,5 mg/ml). Optimization of the purification protocol leads to stable PsbQ protein at required conditions, enabling us to go ahead with getting suitable protein for NMR spectroscopy. NMR spectroscopy implies a nucleus with an uneven spin, and therefore ^{15}N and ^{13}C labelled proteins are needed. Uniform labeling of proteins is typically achieved using minimal media for growing of bacteria, so called M9 media, and we were able to apply our experience gained recently when preparing labelled PsbH protein for similar purposes [Stys *et al*., 2005]. These media contain single nitrogen (amonium chloride, amonium sulfate) and carbon source (glucose). While this arrangement facilitates the straightforward isotopic replacements of these elemements, growth characteristics of *Escherichia coli* are changed compared to growth in rich LB media. Decreasing the growth rate leads to lower yields of the protein. More expensive, however possible it is the overexpression in labelled rich media, which contain a mixture of the isotopicaly-labelled biomolecules. These media are commercially prepared and sold under the brand BioExpress [Rhima *et al*., CIL application]. By use of these media as an additive to minimal media we achieved increased growth rates of *Escherichia coli* and as well as higher yields of proteins. The supplement of 10% Bioexpress is possible to use only in minimal media with istopicaly-labelled nitrogen and carbon. For producing required concentrations of the PsbQ protein with good quality (without precipitation or degradation) it was necessary to optimize growth of B96 cells in M9 medium with labelled and with unlabelled material. Growth characteristics of B96 cells were compared and it was shown that addition of 10% Bioexpress increases the yield of the double labelled PsbQ protein. Finally minimal media [Sambrook *et al.,* 2001] were adjusted by using trace elements [Neidhart *et al*, 1974], thiamine and double labelled M9 media by BioExpress solution [Rhima *et al*., CIL application].

In the case of PsbQ protein, the purification protocol used for unlabelled protein [Balsera *et al*., 2005] did not work for the overexpression with labelled nitrogen

(labelled PsbQ) and labelled nitrogen and carbon (double labelled PsbQ) due to a dramatic decreasing of the yield. Therefore the protocol had to be modified and optimized to gain the required amount of protein with a minimum of loss during purification.

Changes to the PsbQ purification protocol were made at the saturation step and further at the purification step by ionex-exchange chromatography. Precipitation by amonium sulfate is used for purification of soluble proteins. In this step a lot of proteins are lost and PsbQ is no exception. Therefore we tried to purify PsbQ protein without saturation by amonium sulfate. However, purity of the PsbQ protein after ionex-exchange chromatography was not sufficient, indicating that precipitation by amonium sulfate is an important step in the purification protocol. We had to find another possibility to improve the purification protocol. It was shown that saturation by 100% amonium sulfate can precipitate most of the PsbQ protein from the protein solution and it is possible to purify fractions of PsbQ protein from major part of other proteins. The use of an anion-exchange column before cation-exchange column was a helpful step to get pure PsbQ protein. Quality of PsbQ protein was checked by Raman spectroscopy and no secondary or tertiary structure changes were observed. After the preparation of labelled material, experiments were performed at Linz University in the group of Norbert Mueller under supervision of Wolfgang Schoefberger. In the beginning ^{1}H-^{15}N-HSQC measurements were performed to verify that all the ^{15}N-H amide resonances are visible and signals are well resolved in the spectrum (Fig. 14).

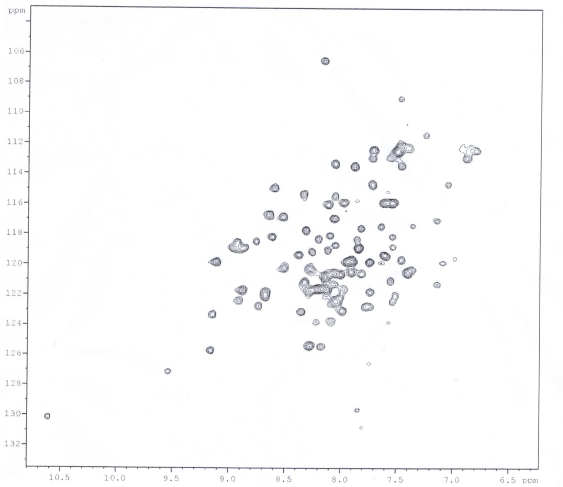


Figure 14. Two dimensional ^{15}N HSQC spectrum of the ^{15}N labelled PsbQ protein

After fulfilling these requirements, NMR experiments for the backbone assignment of the protein were set up. ^{1}H-^{15}N-HSQC, ^{1}H-^{13}C-^{15}N (HNCA, HNCO and HCACO) were run on the double labelled $^{15}N/^{13}C$ PsbQ protein. In the first experiments a few peaks were not well separated, most probably due to the lower field strength of the magnet used in Linz laboratory. Therefore additional experiments were performed in Berlin and Brno, mainly by Michaela Horničáková (Ph.D. student in the group of Prof. Mueller currently working on Protein NMR). These newer spectra can finally be used for the assignment of the single amino acids, which can be used to solve the full protein structure. Up to now more than 80% of the protein backbone was assigned and we expect to get full structural information in a reasonable time [Horničáková *et al.,* 2009].

3.2 Structural analysis of the PsbP protein

The crystal structure of PsbP protein from tabacco, the only available until now, was prepared using recombinant protein overexpressed in *Escherichia coli.* The crystal analysis has shown that the first nine N-terminal residues are degraded [Ifuku *et al.,* 2003] and not present in the crystal, so this part was genetically removed [Ifuku *et al.,* 2004]. As we are working with spinach, the high-resolution structure of spinach PsbP that is as complete as possible is a necessity for detailed atomic simulations of protein-protein interactions. The aim of this part of our research was to prepare the stable recombinant PsbP protein from spinach, which could be used for crystallization and later for interaction studies with recombinant spinach PsbQ protein.

The recombinant protein PsbP from spinach was overexpressed in *Escherichia coli* with a His-tag as HisPsbP protein, subsequently purified and crystallized. The crystal growth of the recombinant PsbP protein without partial degradation and preliminary determination of the crystal structure at the resolution of 2.06 Å is reported [Kohoutová *et al.*, 2009]. Stable recombinant protein PsbP enabled structural studies by other methods (vibrational spectroscopy, NMR, protein-protein crosslinking, surface plasmon resonance, AFM).

The structure of the PsbP protein from spinach was determined by X-ray crystallography, missing parts were computationally modeled, and the structure was

analyzed by vibrational spectroscopy and bioinformatic methods [Kopecký, Kohoutová *et al*., 2010]. Connection of data from X-ray crystallography, Raman spectroscopy of the protein in solution, in the crystal and in the so called „glass phase" (DCDR spectroscopy) as well as molecular modeling demonstrated the power of DCDR spectroscopy as a new, fast and reliable approach in structural biology. With respect to the PsbP, these methods allowed us to analyze the structure and dynamics of the PsbP protein on an atomic level and to propose a convincing model for the full protein structure.

Structural knowledge and hypothetical models of extrinsic proteins PsbP [Kopecký, Kohoutová *et. al*., 2010] and PsbQ [Ristvejová *et al.,* 2006] will be used for the proposal of interaction area of PsbP and PsbQ protein.

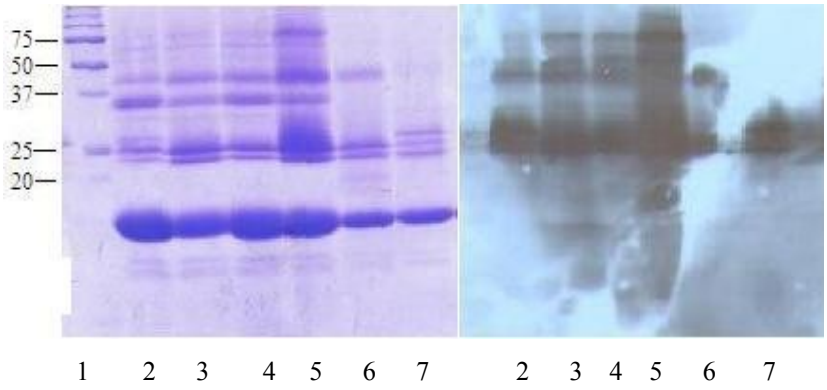
3.3 Interaction studies of extrinsic proteins PsbP and PsbQ

Recombinant PsbP [Kohoutová *et al.,* 2009] and PsbQ [Balsera *et al*., 2005] proteins were used for biochemical analyses. Chemical crosslinking is one of the possibilities to study and analyse interactions between PsbP and PsbQ proteins. This method was used to study interactions of extrinsic proteins in native spinach sample [Frankel *et al*., 1999; Bricker & Frankel, 2003]. The advantage of using recombinant proteins is the ability to study the interaction between two proteins only, without influence of the interaction with other proteins of the whole PSII.

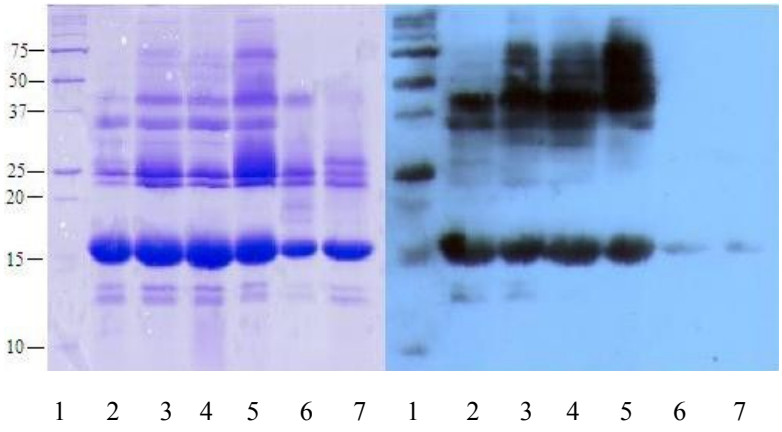
In this study crosslinking reagents such as EDC (covalent bound between -COOH and $-NH_2$), DSS, DSG, DST (covalent bound between two $-NH_2$ groups) were used and a two step crosslinking reaction with Sulfo-SMCC and SATA was performed. It was shown, that recombinant PsbP and PsbQ proteins are not stable in buffers recommended for EDC at pH range from 4.0-6.0. In higher pH self conjugation of the proteins was occured. As the crosslinking with reagents DSS, DSG and DST was negative, the two step crosslinking has been chosen for studying of PsbP-PsbQ interactions.

The first step of this type of crosslinking reaction is activation of one protein by Sulfo-SMCC (to get from $-NH_2$ sulfhydryl reactive maleimide group) and activation of second protein by SATA (to get from $-NH_2$ sulfhydryl group). The second step is the

actual reaction of modified proteins. Control experiment was performed by crosslinking of the each protein by itself. From obtained results it was shown that modified PsbQ protein by Sulfo-SMCC reacts not only with modified PsbP protein by SATA, but also with unmodified PsbP protein (Fig.15). Heterodimers of PsbP/PsbQ, HisPsbP/PsbQ, bound antibodies anti-PsbP and anti-PsbQ and are between the 37 kDa - 50 kDa. From the Western blotting analysis it is visible, that results of crosslinking reactions are not only heterodimers.



a) anti-PsbP (1:9 000)



b) anti-PsbQ (1:10 000)

Figure 15. SDS PAGE (12% acrylamide in separating gel) and Western blotting analysis of crosslinking reactions: a) anti-PsbP: Lane 1. molecular weight marker, Lane 2. PsbQ +SulfoSMCC/ HisPsbP+SATA, Lane 3. PsbQ+SulfoSMCC/ PsbP+SATA, Lane 4. PsbQ+SulfoSMCC /HisPsbP, Lane 5. PsbQ+SulfoSMCC/PsbP, Lane 6. PsbP+SulfoSMCC/ PsbQ+SATA, Lane 7. HisPsbP+SulfoSMCC/ PsbQ +SATA. b) anti-PsbQ: Lane 1. molecular weight marker, Lane 2. PsbQ+SulfoSMCC/ HisPsbP+SATA, Lane 3. PsbQ+SulfoSMCC /PsbP+SATA, Lane 4. PsbQ+SulfoSMCC /HisPsbP, Lane 5. PsbQ+SulfoSMCC/ PsbP, Lane 6. PsbP+SulfoSMCC /PsbQ+SATA, Lane 7. HisPsbP+SulfoSMCC/ PsbQ +SATA

From these results we expected the sulfhydryl groups of PsbP protein and amine group of PsbQ protein to be responsible for the covalent crosslinking of PsbP-PsbQ proteins. PsbP protein contains one sulfhydryl group in Cys159 and sulphur in Met19. We performed crosslinking between PsbQ protein and mutants of PsbP protein: PsbP-PsbQ(Cys159Ala) and PsbP-PsbQ(Met19Ala), but the result showed, that the two step crosslinking reaction is not as specific as we expected and therefore we were not able to determine the type of conjugate and interaction area by this method, only examine the general ability of two proteins to interact. For this reason we designed an experiment that uses affinity chromatography for determining the interaction [Kohoutová *et al*., 2010]. PsbQ protein is not binding to Ni^{+} Sepharose column (affinity column). This property was used to study of interaction between the PsbQ protein and the HisPsbP protein, which binds to Ni^{+} Sepharose column by the His-tag. It was shown, that interaction between these extrinsic proteins are salt dependent and there interactions were characterized by dissociation constant (Kd) determined using AFM and surface plasmon resonance [Kohoutová *et al*., 2010].

4 Outlook

The effect of several amino acids on interactions is evident from the model obtained during our research [Kohoutová *et al*., 2010]. This research will continue by studying of interactions using mutants of extrinsic proteins. Mutations of chosen amino acids will help to indentify amino acids responsible for interactions of PsbP and PsbQ proteins. First screening of interactions will be done using affinity chromatography, surface plasmon resonance will be applied to determine dissociation constant (Kd) and Raman spectroscopy will be used (Trp used like a label) for observation of the vicinity of selected amino acids. Results will be used for better characterization of interactions between extrinsic proteins of the oxygen-evolving complex PSII from higher plants.

5 Abreviations

PSII (P680)	photosystem II
PSII RC	reaction centre of the photosystem II
OEC	oxygen-evolving complex
OEEpps	extrinsic proteins of the oxygen- evolving complex
WOC	water-oxidizing complex
MSP	manganese stabilizing protein
LHC	light harvesting complex
LHCII	light harvesting complex of the photosystem II
QB	reversibly bound plastoquinone
PQH_2	plastohydroquinone
ATP	adenosinetriphosphate
Y_Z	tyrozin
3D	three dimensional
PQ	plastoquinone
BBY membranes	thylakoid membranes (Berthold, Babcock and Yocum)
PMSF	phenylmethanesulfonyl fluorid
EDTA	ethylendiamintetraacetic acid
DMSO	dimethyl sulfoxid
LB medium	Luria Broth medium
M9 medium	minimal medium
EDC	1- Ethyl –3-[3-dimethylaminopropyl]carbodiimide hydrochloride]
DSS	disuccinimidyl suberate
DSG	disuccinimidyl glutarate
DST	disuccinimidyl tartrate
SATA	*N*-succinimidyl-S-acetylthioacetate
Sulfo-SMCC	Sulfo succinimidyl 4-[N/maleimidomethyl]cyklohexane-1-carboxylate]
PPII	polyproline type II structure
MAD	multiwavelength anomalous diffraction
ET	electron transfer

NPQ	non-photochemical quenching
FTIR	Fourier Transform Infrared spectroscopy
CD	Circular Dichroism spectroscopy
NMR	Nuclear Magnetic Resonance
DCDR	Drop Coating Deposit Raman spectroscopy
AFM	Atomic Force Microscopy
X-ray diffraction	Röntgen diffraction
PDB code	protein database code
Kd	dissociation constant
SDS	sodium dodecylsulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis

6 Literature

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65. Instructions for DST, Prod. No. 20589 Doc. No. 0546, Pierce, 1-2
66. Instructions for DSG, Prod. No. 20593 Doc. No. 0543, Pierce, 1-2

7 Appendix

Publications

1. Štys, D., Schoefberger, W., Halbhuber, Z., Ristvejová, J., Mueller, N., Ettrich, R. (2005) Secondary structure estimation of recombinant PsbH, encoding a photosynthetic membrane protein of cyanobaterium *Synechocystis sp.PCC 6803*, *Photosynthetica* 43(3), 421-424.
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*shared first-authorship

Poster presentation

Horničáková, M., Fiala, R., Ristvejová, J., Ettrich, R., Fehlhofer, C., Schoefberger, W., Müller, N., Photosystem II-NMR investigationof the extrinsic PsbQ proteins, 13th Austrian Chemistry Days 2009, August 24-27. 2009, Vienna/Austria

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Secondary structure estimation of recombinant *psbH*, encoding a photosynthetic membrane protein of cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

The PsbH protein of cyanobacterium *Synechocystis* sp. PCC 6803 was expressed as a fusion protein with glutathione-S transferase (GST) in *E. coli* grown on a mineral medium enriched in 15N isotope. After enzymatic cleavage of the fusion protein, the 1H-15N-HSQC spectrum of PsbH protein in presence of the detergent β-D-octyl-glucopyranoside (OG) was recorded on a *Bruker DRX* 500 MHz NMR spectrometer equipped with a 5 mm TXI cryoprobe to enhance the sensitivity and resolution. Non-labelled protein was used for secondary structure estimation by de-convolution from circular dichroism (CD) spectra. Experimental results were compared with our results from a structural model of PsbH using a restraint- based comparative modelling approach combined with molecular dynamics and energetic modelling. We found that PsbH shows 34–38 % α-helical structure (Thr36-Ser60), a maximum of around 15 % of β-sheet, and 12–19 % of β-turn.

Abstrakt

Protein PsbH ze sinice *Synechocystis* sp. PCC 6803 byl exprimován jako fuzní protein s GST (glutatione-S-transferaza) v *Escherichia coli,* které byly kultivovány v minerálním médiu obohaceném isotopem dusíku 15N. Po enzymatickém štěpení fuzního proteinu bylo naměřeno 1H-15N-HSQC spektrum PsbH proteinu v prostředí detergentu β-D-octyl-glucopyranoside (OG) použitím NMR spektrometru *Bruker DRX* 500 MHz s 5 mm TXI cryoprobe pro zvýšení citlivosti a rozlišení. Isotopicky neznačený protein byl použit pro určení sekundární struktury dekonvolucí ze spekter cirkulárního dichroismu (CD). Experimentální výsledky byly porovnány s našimi výsledky strukturního modelu PsbH proteinu získaného použitím metody restraint-based porovnaci modelování kombinovaným s molekulární dynamikou a energetickým modelováním. Struktura PsbH proteinu obsahuje 34–38 % α-helikální struktury (Thr36-Ser60), maximálně 15% β-složeného listu a 12–19 % β-ohybů.

Jaroslava Ristvejová: 10% podíl na publikaci

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Structure and dynamics of the N-terminal loop of PsbQ from photosystem II of *Spinacia oleracea*

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Abstract

Infrared and Raman spectroscopy were applied to identify restraints for the structure determination of the 20 amino acid loop between two β-sheets of the N-terminal region of the PsbQ protein of the oxygen evolving complex of photosystem II from *Spinacia oleracea* by restraint-based homology modeling. One of the initial models has shown a stable fold of the loop in a 20 ns molecular dynamics simulation that is in accordance with spectroscopic data. Cleavage of the first 12 amino acids leads to a permanent drift in the root means square deviation of the protein backbone and induces major structural changes.

Abstrakt

Pro účely modelování (restraint-based homológni modelování) struktury smyčky složené z 20 aminokyselin, která se nachází mezi dvěma β-složenými listy N-koncového regionu PsbQ proteinu (současti kyslík-vyvíjejíciho komplexu fotosystému II) ze *Spinacia oleracea,* byla použita infračervená a Ramanova spektroskopie. Prvotní modelování prokázalo stabilní zbalení smyčky v průběhu 20 ns molekulárne dynamické simulace, což se shoduje se spektroskopickými daty. Odštěpení prvních 12 aminokyselín, vedlo k permanentnímu zvýšení středni kvadratické odchylky struktury polypeptidového řetězce a vedlo k významním struktúrním změnám.

Jaroslava Ristvejová: 30% podíl na publikaci

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Crystallization and preliminary crystallographic characterization of the extrinsic PsbP protein of photosystem II from *Spinacia oleracea.*

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Abstract

Preliminary X-ray diffraction analysis of the extrinsic PsbP protein of photosystem II from spinach (*Spinacia oleracea*) was performed using N-terminally His-tagged recombinant PsbP protein overexpressed in *Escherichia coli*. Recombinant PsbP protein (thrombin-digested recombinant His-tagged PsbP) stored in bis-Tris buffer pH 6.00 was crystallized using the sitting-drop vapour diffusion technique with PEG 550 MME as a precipitant and zinc sulfate as an additive. SDS–PAGE analysis of a dissolved crystal showed that the crystals did not contain the degradation products of recombinant PsbP protein. PsbP crystals diffracted to 2.06 Å resolution in space group $P2_12_12_1$, with unit-cell parameters a = 38.68, b = 46.73, c = 88.9 Å.

Abstrakt

Předběžná rentgenová difrakční analýza vnějšího proteinu PsbP z fotosystému II ze špenátu (*Spinacia oleracea*) byla provedena použitím rekombinantního PsbP proteinu s His kotvou na N-konci, exprimovaného v *Escherichia coli*. Rekombinantní protein PsbP (trombinem naštěpený rekombinantní HisPsbP protein) uchováván v pufru bis-Tris pH 6.00 byl krystalizován použitím metody difuze par v sedící kapce s precipitantem PEG 550 MME a s přídavkem síranu zinečnatého. SDS-PAGE analýza rozpuštěných krystalů ukázala, že krystaly jsou bez částečné degradace rekombinantního proteinu. PsbP krystaly difraktovaly s rozlišením 2.06 Å, prostorové grupy $P2_12_12_1$, s parametry základní buňky a = 38.68, b = 46.73, c = 88.9 Å.

Jaroslava Kohoutová: 50% podíl na publikaci

Can drop coating deposition Raman spectroscopy distinguish shortcomings or inaccuracies in protein crystals? Case study of PsbP protein of photosystem II from *Spinacia oleracea*

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Abstract
Raman crystallography permits collections of data from crystals *in situ* in hanging drops, thus it is used for crystal structure verification in combination with Raman measurements of solvated protein. Nevertheless, even if protein crystal structures seem to be close to the prevailing conformation in solution, Raman difference spectra always show changes in the structures. These changes were ascribed to differences between the structure of the water layer surrounding the protein and/or ligand in solution and the situation in the crystals. However, experimental validation of this presumption that could precisely point on shortcomings or inaccuracies in crystal structures with respect to solvent structures was impossible. Here we apply a new, fast technique of nonresonance Raman spectroscopy – a drop coating deposition Raman (DCDR) method – based on a coffee ring effect that enables nondestructive measurements of solutions with concentration of biomolecules down to 1 μM, in the role of an "arbiter". DCDR protein samples represent a "phase transition" between saturated protein solutions and crystals. Possibilities of this new approach are demonstrated on extrinsic PsbP protein of photosystem II from *Spinacia oleracea* and compared with its crystal structure, that we report at a 1.98 Å resolution. The combination of DCDR and Raman spectra of PsbP in solvent and the crystal points on differences in protein crystal structure with respect to solution and favour the explanation that differences are a density artifact caused by the water envelope. Moreover, based on the structural information gained by Raman spectroscopy, we were able to model two additional loop regions in the protein, that are not resolved in the crystal structure, gaining thus a complete structural picture of the PsbP core.

Abstrakt

V této práci prezentujeme výsledky Ramanové krystalografie, pomocí které byly získány data z krystalu z visící kapky *in situ* a tím bylo možné porovnat krystalovou strukturu v kombinaci s měřením proteinu v roztoku pomocí Ramanové spektroskopie. Přesto, že se zdá, že krystalová struktura je podobná struktuře proteinu v roztoku, Ramanova diferenční spektra ukázali odlišnosti. Tyto odlišnosti popisují rozdíly mezi strukturou proteinu ve vodním prostředí a strukturou proteinu v krystalu. Neexistuje jiná možnost experimentálního ověření, která by popisovala nepřesnosti a nedostatky v krystalu s ohledem na strukturu proteinu v roztoku. V práci byla použita nová a rychlá metoda neresonanční Ramanové spektroskopie – metoda kapkově nanášených povlaků (DCDR), která je založena na interakci molekul a speciálně k tomu připraveného hydrofobního povrchu. Měření lze provádět v nižších koncentracích biomolekul (do 1 μM) než je obvyklé u standardní Ramanovy spektroskopie. DCDR vzorky představují "fázový přechod" mezi nasyceným proteinovým roztokem a krystalem. Možnosti této nové techniky jsou demonstrovány na proteinu PsbP z fotosystému II ze špenátu a porovnány s rentgenovou krystalovou strukturou s rozlišením 1.98 Å. Kombinace výsledků DCDR a Ramanových spekter PsbP proteinu v roztoku a v krystalu a diferenčních spekter umožnilo vysvětlit odlišnosti v strukturách v důsledku vlivu vody. Na základě získaných výsledků bylo možné doplnit informace rentgenové krystalové struktury a tím upřesnit obraz struktury jádra PsbP proteinu.

Jaroslava Kohoutová: 30% podíl na publikaci

Interaction studies of extrinsic proteins PsbP and PsbQ from PSII of Spinacia oleracea.

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Abstract

Direct interaction of the extrinsic proteins PsbQ and PsbP from photosystem II from spinach (*Spinacia oleracea*) is reported. Interactions were studied using recombinant proteins HisPsbP and PsbQ. Affinity chromatography was used to characterize the interaction and its dependance on the environment. Dissociation constants were determined by AFM and surface plasmon resonance. The gained information is used to discuss potential interfaces on basis of the available crystal structures to get a structural picture of the protein-protein interaction.

Abstrakt

V článku popisujeme interakce vnějších proteinů PsbQ a PsbP fotosystému II ze špenátu (*Spinacia oleracea*). Interakce byly studovány použitím rekombinantních proteinů HisPsbP a PsbQ. Pro charakterizaci interakce a její závislosti na prostředí byla použita afinitní chromatografie. Disociační konstanta byla určená použitím metod AFM a povrchová plasmonová rezonance. Získané informace byly použity pro určení možné interakčni plochy na základě přístupných krystalových struktur s cílem získání strukturního modelu námi studované interakce.

Jaroslava Kohoutová: 50% podíl na publikaci

Photosystem II- NMR investigation of the extrinsic PsbQ protein

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Abstract

Photosystem II (PSII) is a multi-protein pigment complex located in the thylakoid membrane of higher plants, green algae and cyanobacteria that uses light energy for the photosynthetic water-splitting reaction to molecular oxygen crucial for life on the earth. One of the proteins of PSII is the 16 kDa **PsbQ protein** which is the aim of our research. PsbQ is the smallest extrinsic protein and can be found on the lumenal surface of PSII in the higher plants and green algae. PsbQ together with the 23 kDa PsbP and 33 kDa PsbO extrinsic proteins form the oxygen evolving complex (OEC) where the photosynthetic water splittig takes place and they are required for maximal rates of O_2 evolution at physiological concentration of inorganic cofactors like e.g. Ca^{2+}, Cl^-, HCO_3^{2-} [1]. The structural analysis [2] and the high-resolution crystal structure of PsbQ protein from PSII of *Spinacia oleracea* indicate proteins two different structure domains: the **C-terminal region** (residues 45-149) folded as a four up-down helix bundle and the fl exible **N-terminal region** (residues 1-44) which is closely packed and contains two parallel beta strands. In spite of the high resolution of the crystal structure no structure assignment of the loop from residues 14-33 („**missing link“**) could be obtained. It is supposed that PsbQ interacts exactly via the N-terminus with the PsbP protein and thus it is involved in binding to PSII [3]. Because of the presumed essential role of the PsbQ-N-terminal region in the protein-protein interactions with other PSII proteins, our work is focused on the PsbQ (*Spinacea oleracea*) NMR assignment that is required for further protein-protein interaction (PPI) studies using NMR spectroscopy. Here, we present the backbone and side-chain **NMR assignment** of the PsbQ protein with complete backbone and nearly complete side-chain **assignment of the missing link**. Additionally, it was possible to **assign 12 of the 13 prolines** of the PsbQ protein sequence.

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Abstrakt

Fotosystém II je proteinovo-pigmentový komplex lokalizovaný v tylakoidní membráně vyšších rostlin, zelených sinic a řas, který využívá sluneční energii pro kyslík-vyvíjející reakci, při které se uvolňuje kyslík potřebný pro život na zemi. Jeden z proteinů tohoto systému je 16 kDa **PsbQ protein,** jehož strukturní analýza je cílem našeho výzkumu. PsbQ protein je vnější protein a nachází se na lumenální straně PSII ve vyšších rostlinách a zelených řasách. PsbQ protein spolu s 23 kDa PsbP a 33 kDa PsbO vnějším proteinem vytvářejí kyslík-vyvíjející komplex, kde probíhá rozklad vody, a jsou odpovědné za maximální uvolnění O_2 při fyziologických podmínkách v přítomnosti anorganických kofaktorů (Ca^{2+}, Cl^-, HCO_3^{2-}). Na základě strukturní analýzy a krystalové struktury PsbQ proteinu s vysokým rozlišením se ukázalo, že PsbQ protein je složen ze dvou strukturně odlišných domén: **C-koncový region** (aminokyseliny 45-149) sbaleny jako čtyři α-šroubovice a flexibilní **N-koncový region** (aminokyseliny 1-44), který obsahuje dva paralelní β-skládané listy. I přes vysoké rozlišení rentgenové krystalové struktury není možné určit strukturu smyčky v oblasti 14-33 (tzv.**chybějící část**). Předpokládá se, že právě N-koncovým regionem se PsbQ protein váže k PsbP proteinu a teda k PSII. Z tohoto důvodu se náš výzkum zaměřil pravě na analýzu struktury N-koncové oblasti PsbQ proteinu, která je potřebná pro další studium interakci PsbQ a PsbP proteinů pomocí NMR spektroskopie. V práci předkládáme **NMR assignment** PsbQ proteinu s kompletním hlavním polypeptidovým řetězcem a skoro kompletním vedlejším řetězcem s tzv. chybějící části, v které jsme určili **12 ze 13 prolinů** PsbQ proteinové sekvence.

Jaroslava Ristvejová: 5 % podíl