5 Membrane-extrinsic subunits

The oxygen-evolving system of cyanobacteria contains three extrinsic proteins on the lumenal side of the thylakoid membrane, namely, a 33 kDa protein (PsbO), cyt *c*-550 (PsbV), and a 12 kDa (PsbU) protein (Shen and Inoue, 1993). It is not known whether one of these subunits binds organic or inorganic cofactors except for the haem of cyt *c*-550. The 33 kDa protein is commonly found in higher plant and cyanobacterial PSII and its function has been studied extensively. The other two proteins, cyt *c*-550 and the 12 kDa protein, however, are present only in algal-type PSII but absent in higher plant PSII and are functionally replaced by PsbP and PsbQ in higher plants. Cyt *c*-550 is required for maintaining both the oxygen evolution and PSII stability. Nevertheless, interestingly a *psbP* gene is found in the genome of *T. elongatus* and the PsbP and PsbQ proteins were recently identified in *Synechocystis* PC6803 (Thornton *et al.*, 2004). Under calcium or chloride deficient conditions, the deletion mutants Δ*psbP* and Δ*psbQ* showed decreased growth relative to wild type cells (Thornton *et al.*, 2004). These membrane-extrinsic proteins are thought to function the stability and activity of the catalytic active site (Shen *et al.*, 1995; Seidler, 1996).

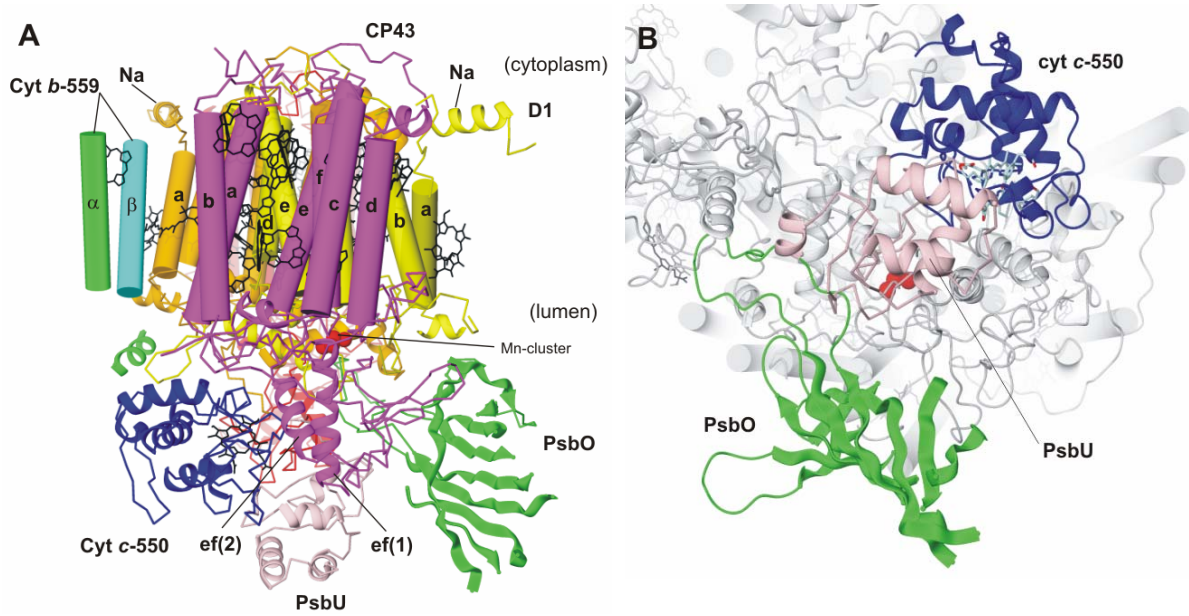

Fig. 5.1: (A) View along the membrane plane of PSIIcc monomer. Assigned subunits in magenta (CP43), yellow (D1), orange (D2), red (CP47), green and cyan (cyt *b*-559, α and β). Unassigned TMH are omitted for clarity. Membrane-extrinsic subunits in green (PsbO), pink (PsbU) and blue (cyt *c*-550). (B) View from the lumenal side: colouring same as in (A). Except for the membrane-extrinsic subunits, all other subunits and cofactors are shown in grey.

5.1 PsbO- 33 kDa protein

By MALDI-TOF MS a molecular weight of 26.83 kDa was determined; assuming an N-terminal presequence of 26 residues, the calculated mass for mature PsbO lacking these residues is 26.82 kDa, in good agreement with the observed peak (Kern *et al.*, 2004b).

PsbO shows a moderate similarity of 40-50% between cyanobacteria and higher plants (Seidler, 1996), even though the cyanobacterial protein reveals deletions and insertions in the primary structure (Bricker and Frankel, 1998). Limited proteolysis of PsbO isolated from different organisms results in different degradation products (Tohri *et al.*, 2002). Structural predictions based on UV-CD spectroscopy (Xu *et al.*, 1994; Lydakis-Simantiris *et al.*, 1999; Motoki *et al.*, 2002) and Fourier transform infrared spectroscopy (Ahmed *et al.*, 1995; Sonoyama *et al.*, 1996; Shutova *et al.*, 1997; Lydakis-Simantiris *et al.*, 1999; Svensson *et al.*, 2004), suggested a high *β*-sheet content of isolated PsbO, whereas other spectroscopic studies suggested a "natively unfolded" structure (Lydakis-Simantiris *et al.*, 1999), and PsbO exhibits pronounced pH-dependent structural changes (Shutova *et al.*, 1997; Weng *et al.*, 2004). These results and other typical features of PsbO were discussed by Shutova *et al*. suggesting a "molten globule" structure with a hydrophobic *β*-sheet structure (Shutova *et al.*, 2000). Computer-assisted modelling proposed PsbO to adopt a double *β*-sandwich fold (Pazos *et al.*, 2001), in obvious disagreement to X-ray data.

Several functions were proposed for PsbO: it affects the stability of the redox-active Mn-Ca-cluster and is therefore also referred to as manganese-stabilizing protein (MSP). PsbO does not directly ligate the Mn-Ca-cluster {Ferreira, 2004 #1541;Kamiya, 2003 #1180;Zouni, 2001 #1516;Loll, 2005 #1601}. Deletion of *psbO* in *Synechocystis sp* PCC6803 reduced the level of oxygen evolution and resulted in a light-sensitive PSII (Mayes *et al.*, 1991; Komenda and Barber, 1995; Burnap *et al.*, 1996). PsbO can be removed from cyanobacterial PSII at high $CaCl_2$ concentrations connected with the loss of two manganese ions of the Mn-Ca-cluster, and most of the oxygen evolving activity could be restored under non-physiological Ca^{2+} and Cl^- concentrations (Miyao and Murata, 1983). After removal from PSII, PsbO remains stable in solution. Rebinding of PsbO to PsbO-deficient PSII partially restores oxygen-evolution and stability. It is generally believed that PsbO provides an optimal ionic environment to the oxygen evolving complex, especially the correct concentration of calcium and chloride ions. For thermophilic cyanobacteria a stabilizing role of PsbO on PSII was proposed connected

with an increased thermostability of the whole complex (Lydakis-Simantiris *et al.*, 1999; Pueyo *et al.*, 2002) because the oxygen evolving activity was strongly diminished after extraction of the membrane-extrinsic lumenal subunits and light-induced peroxide formation was observed (Hillier and Wydrzynski, 1993). These findings led to the hypothesis that PsbO is involved in regulating the accessibility of water to the active site of the water oxidizing complex (Hillier and Wydrzynski, 1993).

The N-terminus of PsbO was predicted to be important for its functional binding to PSII (Eaton-Rye and Murata, 1989). This proposal was substantiated and extended by crosslinking that showed the Glu1-Lys76 domain of MSP to interact with the Glu364-Asp440 domain of the large membrane-extrinsic **ef** loop domain of CP47 (Odom and Bricker, 1992). More evidence for the importance of the N-terminal domain was provided by truncation of five or six amino acids of the N-terminus that had no negative effect on recovery of the oxygen evolution. However, deletion of seven residues decreased reconstitution activity to 40% of the control value and reduced functional binding of the variant protein (Popelkova *et al.*, 2003).

At the current stage of the PSIIcc model, PsbO is modelled as poly-alanine. The poor quality of the electron density made any sequence assignment impossible. The utilisation of a broad variety of secondary structure prediction programs did not assist to overcome this problem.

PsbO forms a hollow cylinder featuring a length of 35 Å and a diameter of 15 Å that is mainly formed by *β*-sheets resembling a *β*-barrel like structure. The high content of β-sheet is in good agreement with CD- and FTIR-spectroscopy {Lydakis-Simantiris, 1999 #334;Motoki, 2002 #829;Xu, 1994 #940;Lydakis-Simantiris, 1999 #334;Shutova, 1997 #525;Sonoyama, 1996 #534;Ahmed, 1995 #3;Svensson, 2004 #1558;Loll, 2005 #1635}. The interactions with the lumenal loop regions of the membrane-intrinsic subunits are mainly performed by two loop region protruding from the core of the β-barrel of PsbO as supported by crosslinking studies (Enami *et al.*, 1991; Odom and Bricker, 1992). The loops are different in length: one being approximately 40 amino acids long and the other of approximately 17 amino acids and both penetrate in the extended loop domain **ef** of CP47.

PsbO is in immediate vicinity to membrane-extrinsic subunit PsbU. Interactions are formed between amino acids located on the exterior of the β-barrel. The opening of the cylinder is directed towards the Mn-Ca-cluster, suggesting a possible channel-like function providing the

active site with the substrate (H_2O). The observation that only two molecules of H_2O occur at the active site, demands for regulation. This could be achieved by flexible loop acting as a lid on the opening of the channel. As the resolution is too low to identify possible water molecules, a possible function as a water channel is at present pure speculation and needs higher resolution data to be verified. The proposed water channel is in contrast to the recent PsbO model of Ferreira *et al.* (2004) as they modelled PsbO with an hydrophobic interior and hydrophilic exterior.

The PsbO subunit contains two conserved cysteine residues: Cys112 and Cys135, which are reported to form a disulfide bridge that is essential for the function of PsbO (Tanaka and Wada, 1988). An anomalous signal for the disulfide bridge was not observed in our electron density maps. Therefore this additional information of the primary sequence could not be used.

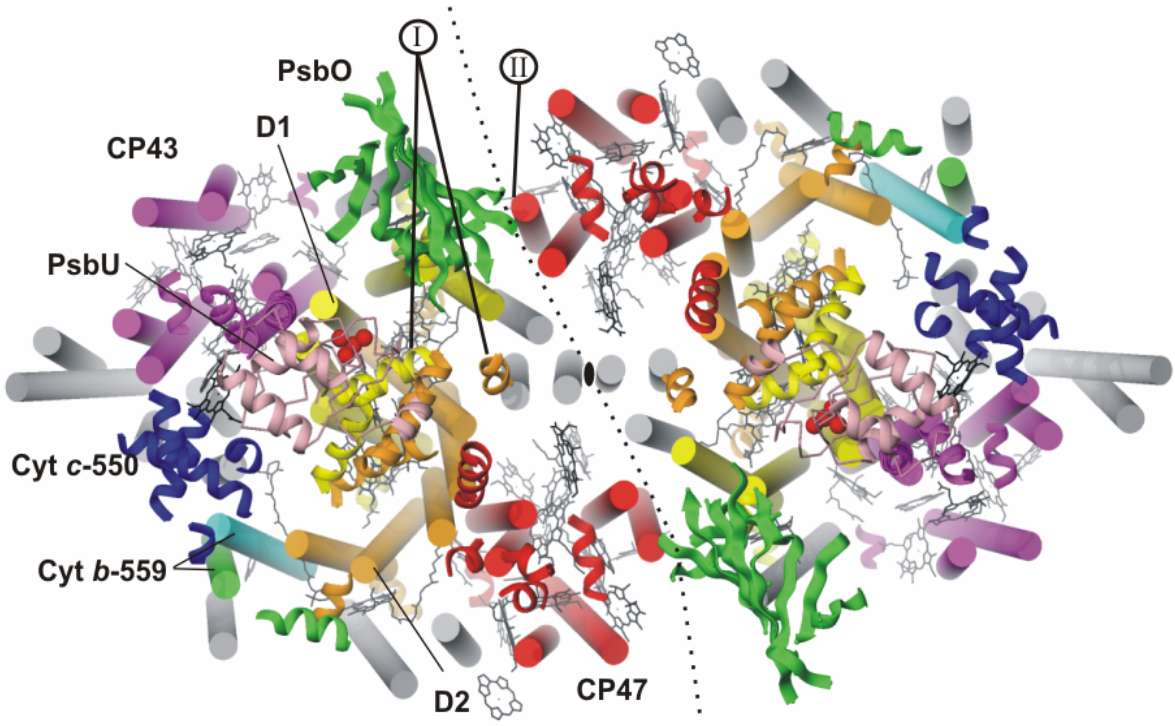

Fig. 5.2: Overall view of PSIIcc dimer from the lumenal side. Non-helical segments are not shown. One monomer is related by the pseudo-*C2* axis (black ellipse) to the other monomer. Assigned subunits in magenta (CP43), yellow (D1), orange (D2), red (CP47), green and cyan (cyt *b*-559, α and β). Membrane-extrinsic subunits in green (PsbO), pink (PsbU) and blue (cyt *c*-550). Unassigned TMH (grey) Chl*a*, haem and Car are drawn in black. The dashed line indicates the monomer-monomer interface. Encircled numbers I and II indicate interaction with lumenal loop-regions of D1 of one monomer and with the extended lumenal loop-region of CP47 of the other monomer.

PsbO interacts with the both monomers of the dimeric PSIIcc complex (Fig. 5.2). The lumenal loop region of D1 of one monomer interacts with PsbO as well as the extended lumenal domain of CP47 of the other monomer. PsbO forms a clamp and stabilises the dimeric organisation of PSIIcc. This appears to be an important function of PsbO and may explain the increased thermostability of the whole complex in thermophilic cyanobacteria (Lydakis-Simantiris *et al.*, 1999; Pueyo *et al.*, 2002) compared to higher plant PSII.

Enami and co-workers (Enami *et al.*, 1998) could demonstrate the rebinding of an intramolecular crosslinked PsbO. They were able to retain full rebinding but its reactivating ability was lost. This indicates that possibly the crosslinker molecule inhibited or reduced homodimerisation of PSII. These specific interactions of PsbO with both monomers might be the reason for the controversies concerning the number of PsbO copies per PSII dimer.

Due to the mentioned problems to model PsbO bound to PSIIcc, attempts were initiated to determine the structure of PsbO in solution. A DNA-construct of PsbO was designed in which the region encoding for the N-terminal signal sequence was truncated. The fragment was cloned into the expression vector pET11a (Novagen). The latter was transformed into *Escherichia coli* strain BL21(DE3) (Novagen). Recombinant PsbO from *T. elongatus* was expressed in *E. coli* and biochemically characterised. A detailed description of all applied microbiological and biochemical methods and results would exceed the scope of this thesis. For a detailed description see Loll *et al.* {Loll, 2005 #1635}

We have compared the UV-CD and FTIR spectra of wild type PsbO isolated from PSIIcc in the presence of urea with those of recombinant PsbO. On the basis of these spectroscopic data, we conclude that recombinant PsbO is correctly folded and can be reconstituted to the native PSIIcc. To elucidate probable Ca^{2+} binding site(s), prior to crystallisation experiments, PsbO was titrated with $CaCl_2$. As no significant changes in the secondary structure could be observed with UV-CD and FTIR spectroscopy, we conclude that cyanobacterial PsbO does not contain a specific binding site for Ca^{2+} {Loll, 2005 #1635}. Furthermore we suggest that PsbO from higher plants might have a Ca^{2+} binding site, leading to a possible structural and functional divergence between the two related polypeptides.

5.2 PsbU

The gene of *psbU*, encodes a polypeptide of 131 residues (15.0 kDa). The first 30 residues serve as a transit peptide required for the transport of PsbU into the thylakoid lumen and which were absent in the mature protein and thus, resulting in an 11.7 kDa protein. PsbU is the smallest membrane-extrinsic subunit. It is not known whether PsbU binds any cofactor.

A *psbU* gene deletion mutant showed that the 12 kDa protein is not essential for oxygen evolution but may play a role in optimizing the ion (Ca^{2+} and Cl^{-}) environment and maintaining a functional structure of the cyanobacterial oxygen-evolving complex. In addition, a double deletion mutant lacking cyt *c*-550 and PsbU grew photoautotrophically with a phenotype identical to that of the single deletion mutant of cyt *c*-550 {Shen, 1997 #517;Loll, 2005 #1635}. It was also shown that the 12 kDa protein cannot bind to photosystem II in the absence of cyt *c*-550 (Shen and Inoue, 1993).

Biochemical studies revealed that cyt *c*-550 and PsbU stabilise the oxygen-evolving machinery against heat-induced inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002 (Nishiyama *et al.*, 1994; Nishiyama *et al.*, 1997). Furthermore, PsbU is required not only for enhancement of the thermal stability of the oxygen-evolving machinery but also for development of cellular thermotolerance, both of which occur during the acclimatisation of cells to high temperature (Nishiyama *et al.*, 1999). These observations suggest that stabilisation of the oxygen-evolving machinery by the membrane-extrinsic proteins is important to establish cellular thermotolerance. By inactivating the genes for these proteins individually, it was demonstrated that the three extrinsic proteins stabilise the oxygen-evolving machinery independently against high-temperature stress (Kimura *et al.*, 2002).

PsbU is located in between PsbO and PsbV (Fig. 5.2). It was not possible to assign the primary sequence as the electron density maps did not allow to trace side chains of amino acids. Therefore no more than secondary structure elements could be modelled in the electron density. The main secondary structure elements of PsbU are five short α-helical patches (Fig. 5.3).

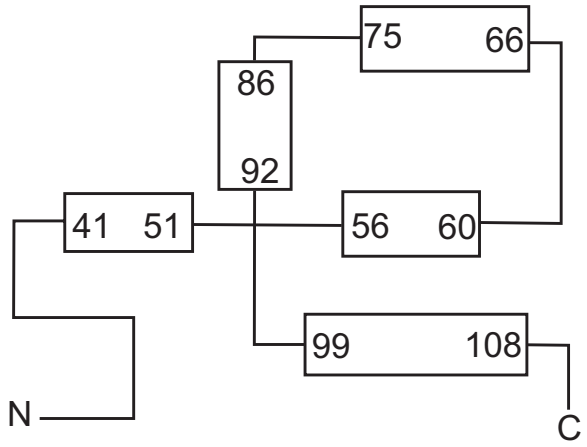

Fig. 5.3: Topography of PsbU: α-helices are indicated by rectangles denoted **α1** to **α5**.

The N-terminus and α1 interacts with the long extrinsic loop between membrane extrinsic α-helices **ef(1)** and **ef(2)** of CP47 anchoring PsbU to the PSIIcc (Fig. 5.4). The central globular domain is formed by short α-helices α2 to α5. The loop between α4 and α5 interacts with the loop between TMH-**a** of CP47 and the α-helical fragment **ab(1)** of CP47 as well as the C-terminus of CP47 points towards the C-terminus of PsbV.

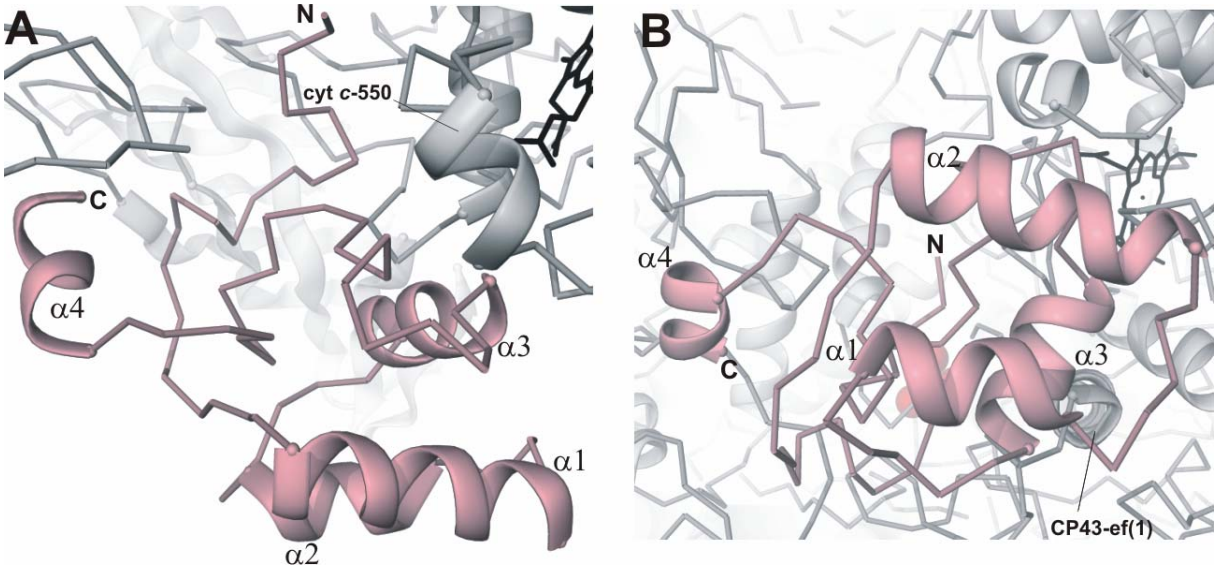

Fig. 5.4: Membrane-extrinsic PsbU drawn in pink and other protein subunits are indicated in grey. (**A**) Side view onto the membrane plane. (**B**) View from the lumenal to the cytoplasmic side.

5.3 PsbV - cytochrome *c*-550

The *psbV1* gene product cyt *c*-550 is a 18.0 kDa protein (Shen and Inoue, 1993; Kerfeld and Krogmann, 1998; Shen *et al.*, 1998). After processing, the protein has a molecular weight of 15.8 kDa bearing a covalently bound haem-group. The midpoint potential of cyt *c-550* is unusually low for a c-type cytochrome; approximately -240 mV at pH 6 (Navarro *et al.*, 1995; Roncel *et al.*, 2003). Recent biochemical and EPR studies revealed that the redox potentials and the EPR characteristics of the soluble and PSII-bound forms of *T. elongatus* cyt *c*-550 are markedly different (Vrettos *et al.*, 2001; Roncel *et al.*, 2003). Redox potentials of -240 mV and -80 mV and at pH 6.0 were reported for PSII-bound and isolated PsbV (Roncel *et al.*, 2003). In cyanobacteria PsbV is lumenally associated with PSII where it appears to stabilise the oxygen-evolving complex (Nishiyama *et al.*, 1994) and it is close to cyt *b-559*, CP43 and PsbU (Fig. 5.2). PsbV is predominantly α-helical with two short anti-parallel β-sheets (Fig. 5.5).

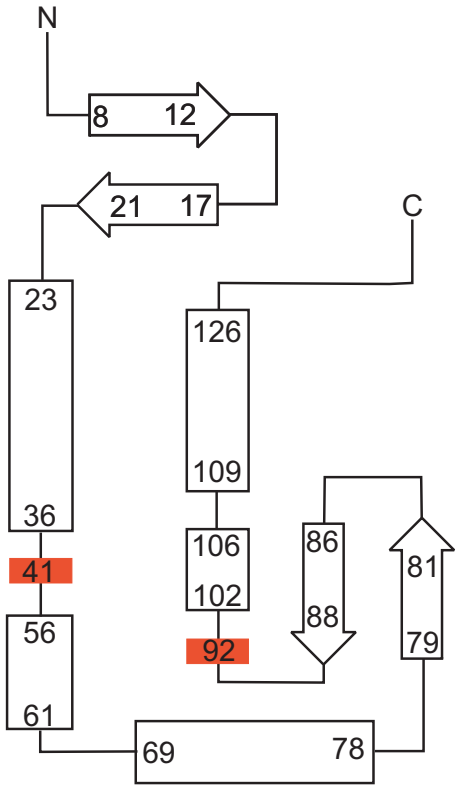

Fig. 5.5: Topography of cyt *c*-550: α-helices are indicated by rectangles denoted α1 to α5 and β-sheets by arrows. Haem coordinating histidine residues are marked with red background.

The N-terminus of PsbV points towards the loop region between TMH-**e** and the membrane attached α-helix **eC** of D1 as well as to the α-chain of cyt *b*-559. An H-bond is formed

between the backbone oxygen of PsbV-Glu2 and PsbV-Thr4 and PsbE-Gln58 located on the lumenal loop of the α-chain of cyt *b*-559. A two-stranded β-sheet (β1 and β2) is found near the N-terminus followed by α-helix (α1) in the neighbourhood of CP43. Close to the Fe^{2+} coordinating residue PsbV-His41, located on a short loop segment between α1 and α2 (Fig. 5.5), a H-bond is formed from PsbV-Asn49 to CP43-Arg320, located on **ef(1)** of CP43 (Fig. 4.16).

It should be noted that the amino acid sequence is not fully assigned in the extended loop region of CP43. Therefore the number of inter-subunit interactions is restricted to the current model. N-terminal of the second antiparallel β-sheet (β3 and β4), α-helix (α3) is located. β3 and the short loop linking β3 and β4 is in contact with the N-terminal part of PsbU. The loop between β4 and α4 is close to **ef(3)** in the extended loop of CP43 (Fig. 4.16). PsbV-His92, coordinating the Fe^{2+} is located downstream of two short α-helices (α4 and α5). The backbone oxygen of PsbV-Ile125 in the last turn of α5 is involved in H-bonding to D1-Asn312. The C-terminus of PsbV is in close vicinity (~8 Å) to the currently modelled C-terminus of PsbU.

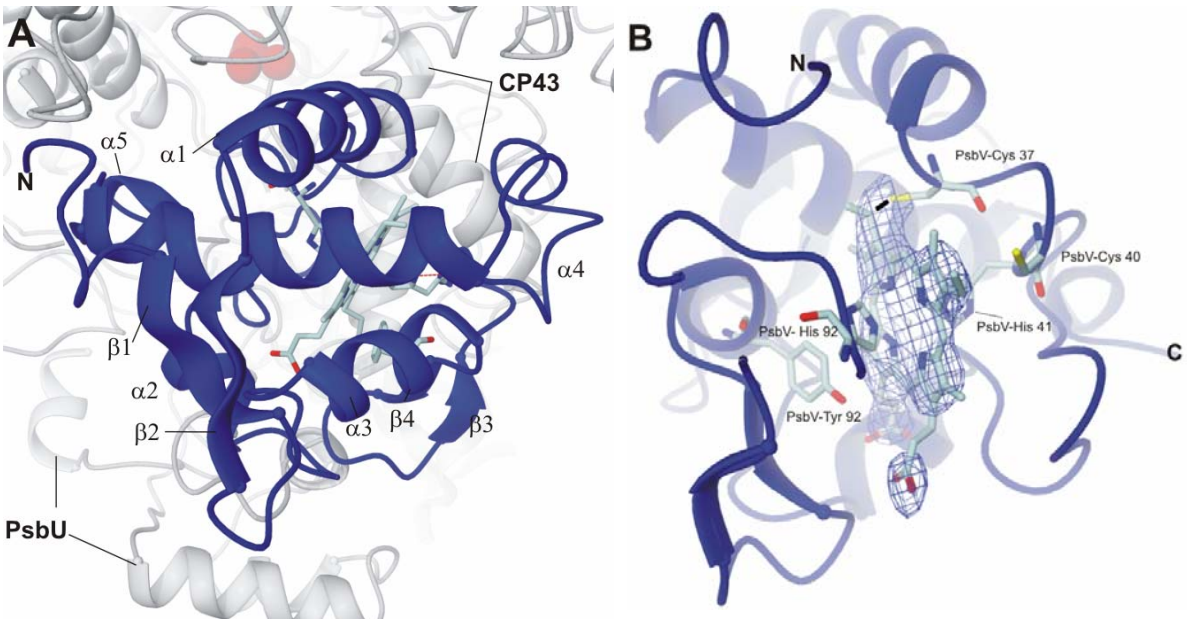

Fig. 5.6: Representation of cyt *c*-550 (dark blue): (**A**) View onto the membrane plane. The haem and coordinating histidines are shown. All other subunits and cofactors are shown in grey. (**B**) Cyt *c*-550 is coordinated by PsbV-His41 and PsbV-His92. A thioether bond is formed within the haem vinyl group $C3^1$ of the haem and PsbV-Cys37 (black bar). Electron density (blue) around the haem is contoured at 1.0 σ level.

The haem group of PsbV is well defined in the electron density, tilted by 62° to the membrane plane (angle between the heterocycle plane normal and the pseudo-*C2*(Fe^{2+}) axis) and shielded from solvent by hydrophobic residues of PsbV and the extended lumenal domain of CP43 (Fig. 5.6A). Fe^{2+} is coordinated by PsbV-His41Nε and PsbV-His92Nε (Table 3) that is H-bonded with NδH to PsbV-Pro92O (2.7 Å). The two haem propionate carboxy groups are not in direct contact with protein and probably hydrated. The hydrophobic environment of the haem and the close distance of PsbV-Tyr75Oη···Fe^{2+}, 5.0 Å, could modulate the haem redox properties (Fig. 5.6B). The redox potential difference between bound and isolated PsbV (-240 mV and -80 mV, respectively at pH 6.0) (Roncel *et al.*, 2003) could be caused by the additional shielding of the haem group by CP43 in the bound state. PsbV-Cys37 of the ^{37}CysXXCysHis^{41} motif (Barker and Ferguson, 1999), a common feature of *c*-type cytochromes, forms a thioether bond with the haem vinyl group $C3^1$, whereas PsbV-Cys40 is close enough to vinyl group $C8^1$ to also form such a bond, but there is no connecting electron density. The SH group of Cys40 is rotated by ~120 ° about the Cα-Cβ bond and clearly not in the correct conformation to allow such thioether bond (Fig. 5.6B). This could be explained by radiation damage as the MALDI-TOF MS clearly revealed the mass for cyt *c*-550 with covalently attached haem group (Kern *et al.*, 2004b) In the solution structure of cyt *c*-550 the C-terminal region is highly disordered, whereas in the PSIIcc structure, it is stabilised upon binding to the complex points towards the Mn-Ca-cluster. The phenolic PsbV-Tyr137Oη is at a distance of ~11 Å from the Mn-Ca-cluster (Mn56). The two C-terminal tyrosine residues (PsbV-Tyr136 and PsbV-Tyr137) could be of functional importance in possible electron transfer reactions.

The structures of free (Kerfeld *et al.*, 2003) and PSII-bound cyt *c*-550 are very similar (Fig. 5.7), with different conformations found for only few amino acid side chains located at the interface formed by cyt *c*-550 with D1, CP43, and PsbU and the six C-terminal amino acids. The soluble cyt *c*-550 (PDB entry 1MZ4) superimposes on PsbV associated to PSIIcc with an rmsd of 0.3 Å for 131 Cα atom pairs.

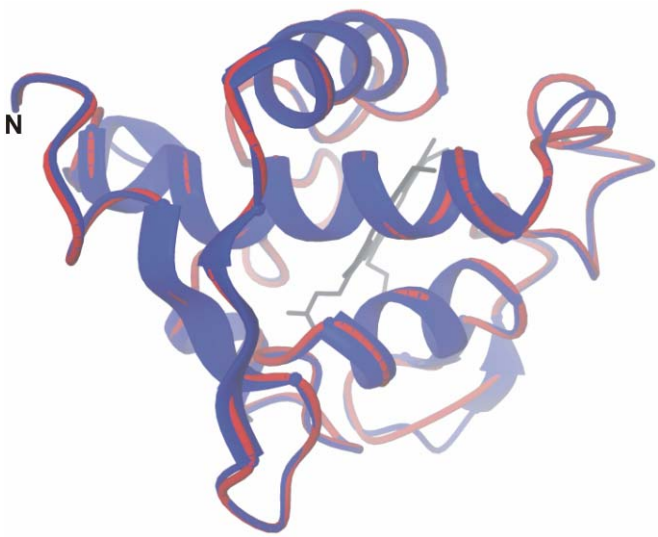

Fig. 5.7: Superposition of free (red) and PSIIcc-bound (blue) cyt *c*-550. Same view point as in Fig. 5.6.