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Institute of Physical Biology

Localization and function of small protein subunits of the cyanobacterial Photosystem II complex

Ph.D. Thesis

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Annotation: The Photosystem II complex (PSII) contains besides D1, D2, CP43, CP47 and several lumenal extrinsic proteins also a large number of small intrinsic subunits. These subunits are found in all kinds of oxygenic phototrophs and their sequences are usually highly conserved. It is assumed that the small subunits are involved in stabilization, assembly or dimerization of the PSII complex but their exact function remains largely unknown. Thus, we chose four small PSII proteins: cytochrome b-559, PsbH, PsbI and Psb28, and we studied their function and location in PSII of the cyanobacterium *Synechocystis* sp. PCC 6803. We attempted: i) to better define the role of cyt b-559 in the assembly of PSII using various mutant strains lacking one or more PSII core protein subunits; ii) to clarify the location of the PsbH subunit using NTA gold labelling methodology; iii) to improve the available knowledge on function of the PsbI protein in the assembly and repair of the cyanobacterial PSII; and iv) to confirm the presence of the Psb28 protein in PSII, to localize it and to establish its importance for the cyanobacterial PSII.

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This dissertation was mostly elaborated at the Laboratory of Photosynthesis, Department of Autotrophic Microorganisms, Institute of Microbiology Academy of Science of the Czech Republic, Opatovický mlýn, 379 81 Třeboň, Czech Republic

List of enclosed publications

This thesis is based on the following publications:

I. **Komenda J, Reisinger V, Müller BC, Dobáková M, Granvogl B, Eichacker LA** (2004) Accumulation of the D2 protein is a key regulatory Stepp for assembly of the photosystem II reaction center complex in *Synechocystis* PCC 6803. *JBC* **279**, 48620-48629.

II. **Bumba L, Tichý M, Dobáková M, Komenda J, Vácha F** (2005) Localization of the PsbH subunit in photosystem II from the *Synecocystis* 6803 using the His-tagged Ni-NTA Nanogold labeling. *Journal of structural biology* **152**, 28 - 35

III. **Dobáková M, Tichý M and Komenda J** (2007) Role of the PsbI protein in Photosystem II assembly and repair in the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Physiology* **145**, 1681 - 1691

IV. **Dobáková M, Tichý M and Komenda J** (2008) The Psb28 protein is preferentially associated with the Photosystem II subcomplex lacking CP43 and participates in synthesis of chlorophyll-binding proteins in the cyanobacterium *Synechocystis* sp. PCC 6803. *Manuscript*

These publications will be referred in the text by the above Roman numerals.

Declaration

I declare that my role in preparation of publications was following:

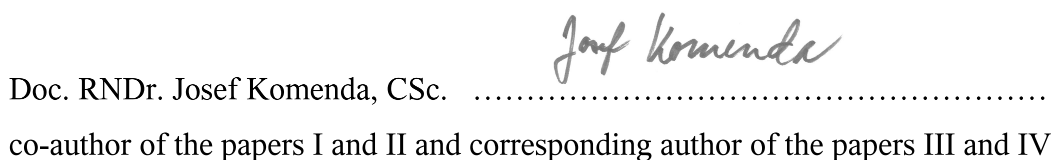
Paper I. Co-author – preparation of the *psbC/psbB* double mutant of *Synechocystis* and its analysis by 2D electrophoresis, identification of the alpha subunit of cytochrome b-559 in PSII complexes of various *Synechocystis* strains by 2D western blot analysis, the estimated overall contribution to the publication 15%

Paper II. Co-author – isolation and purification of PSII complexes form the *Synechocystis* strain expressing the His-tagged PsbH protein, its analysis by electrophoresis and western blot, the estimated overall contribution to the publication 20%

Paper III. Main author – preparation of all *psbI* deletion mutants tested in the publication, their complete characterization, preparation of the manuscript, the estimated overall contribution to the publication 75%

Paper IV. Main author – preparation of all *psb28* deletion mutants tested in the publication, their complete characterization, preparation of the manuscript, the estimated overall contribution to the publication 75%

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



Doc. RNDr. Josef Komenda, CSc. ……………………………………………
co-author of the papers I and II and corresponding author of the papers III and IV

In Nové Hrady, 16.5.2008

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Abbreviations

cyt b-559	Cytochrome b-559
D1	Maturated D1 protein
iD1	Intermediate of D1
pD1	Precursor of D1
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
Q_A, Q_B	Primary and secondary plastoquinone acceptor of photosystem II, respectively
RC	PSII reaction center
RCC	PSII core complex
RCC(1)	PSII monomeric core complex
RCC(2)	PSII dimeric core complec
RC47	PSII core complex lacking the CP43 protein
Synechocystis	*Synecocystis* sp. PCC 6803

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1. INTRODUCTION

1.1. Foreword

Sunlight plays a much larger role in our sustenance than we may expect: all the food we eat and all the fossil fuel we used is a product of photosynthesis, which is the process that converts energy in sunlight to chemical forms of energy that can be used by biological systems. Photosynthesis is carried out by many different organisms, ranging from plants to bacteria (Vermaas, 1998; Nelson and Ben-Shem, 2004).

All photosynthetic organisms that produce oxygen have two types of reaction centers (RC), named photosystem II (PSII) and photosystem I (PSI), pigment-protein complexes that are located in specialized membranes called thylakoids. In eukaryotes (plants and algae), these thylakoids are located in chloroplasts (organelles in plant cells) and often are found in membrane stacks (grana). Prokaryotes (bacteria) do not have chloroplasts or other organelles, and photosynthetic pigment-protein complexes either are in the membrane around the cytoplasm or in invaginations thereof (as is found, for example, in purple bacteria), or are in thylakoid membranes that form much more complex structures within the cell (as is the case for most cyanobacteria). All chlorophyll in oxygenic organisms is located in thylakoids, and is associated with PSI, PSII, or with antenna proteins feeding energy into these photosystems (Vermaas, 1998).

1.2. Photosynthesis

Plant photosynthesis is accomplished by a series of reactions that occur mainly, but not exclusively, in the chloroplast. The chloroplast thylakoid membrane is capable of light-dependent water oxidation, NADP reduction and ATP formation. These reactions are catalyzed by two separate photosystems (PSI and PSII) and an ATP synthase. The electron transport between PSII and PSI is mediated by cytochrome b_6f complex (Barry et al., 1994; Thidholm et al., 2002; Nelson and Ben-Shem, 2004).

Surprisingly, structural comparison of RC from different photosynthetic systems showed that these RC are basically similar to each other in terms of their overall three-dimensional structure (Vermaas, 1998).

Photosynthetic electron transport and photophosphorylation are carried out by large protein-pigment complexes in the thylakoid membranes of cyanobacteria and chloroplasts, and these complexes contain some of the most abundant proteins in nature. Despite their abundance and complexity (PSII alone contains over 25 different polypeptides) very little is known about their assembly (Mann et al., 2000).

PSI and PSII contain chlorophylls and other pigments that harvest light and funnel the excitation to the primary electron donors, which can reduce an electron acceptor and accept electrons from specific electron donors. Most of the chlorophylls that are associated with PSII in higher plants are harboured in the extrinsic, peripheral light-harvesting antenna complex II (LHCII) (Nelson and Ben-Shem, 2004). Phycobilisomes, the major light harvesting antennae of cyanobacteria, are attached to the stromal side of the thylakoids and they primarily transfer excitation energy to PSII (Gantt, 1994; Wollman et al., 1999).

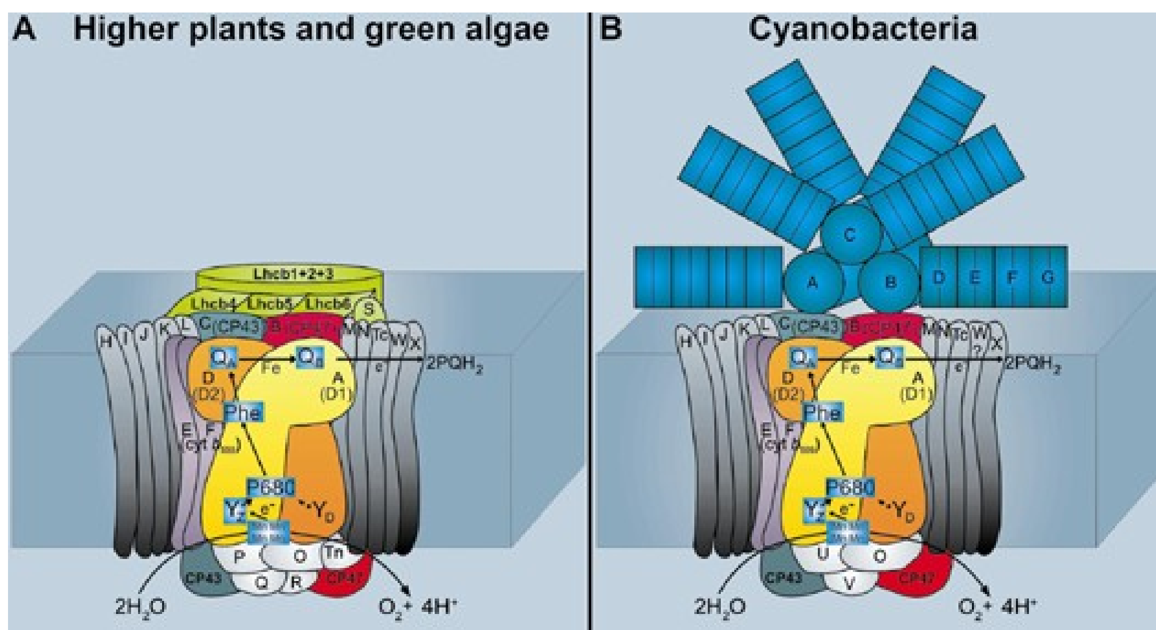

Fig. 1 The structure and subunit composition in A) higher plants and green algae B) cyanobacteria. The arrows shows electron transfer pathway from water oxidation to plastoquinone reduction. From Hankamer et al., 2001

1.3. Structure and function of PSII complex

PSII is a multisubunit protein-cofactor complex embedded in the thylakoid membrane of higher plants, algae and cyanobacteria and drives the water oxidation process of photosynthesis, which split water into reducing equivalents and molecular oxygen using solar energy (Hankamer et al., 1997; Mattoo et al., 1999; Thidholm et al., 2002; Shi and Schröder, 2004; Loll et al., 2005). PSII mostly exists as a dimer (Hankamer et al., 2001; Thidholm et al., 2002) consisting of almost 25 different subunits encoded by both plastid and nuclear DNA (Shi et al., 2000).

At the heard of the PSII complex there is a RC heterodimer performing primary charge separation (Nanba and Satoh, 1987; Zheleva et al., 1998; Wollman et al., 1999; Zouni et al., 2001; Barber, 2006) and consisting of two homologous proteins known as D1 (*psbA* gene product) and D2 (*psbD* gene product). The D1 and D2 subunits bind most of the cofactors needed for primary and secondary electron transfer reactions and show a high degree of similarity to the L and M subunits of the RC of purple bacteria (Nanba and Satoh, 1987; Tomo et al., 1993; Barber, 1998; Wollman et al., 1999; Diner and Rappaport, 2002). The D1-D2 heterodimer is closely associated with the chlorophyll *a* binding proteins, CP47 and CP43 (Salih and Jansson, 1998; Zheleva et al., 1998; Shi et al., 2000; Zouni et al., 2001). These proteins act both as "inner" light harvesting system transferring the captured energy by an "outer" light harvesting system composed either of proteins that bind chlorophyll *a* and *b* (in the case of higher plants and green algae) or phycobilisomes (in the case of cyanobacteria and red algae) (Zheleva et al., 1998) to the chlorophylls in the PSII RC. These consists of two weakly coupled chlorophylls P_A (P_{D1}) and P_B (P_{D2}) and two accessory chlorophylls, B_A (B_{D1}) and B_B (B_{D2}) (Diner and Rappaport, 2002). The latest data (Diner and Rappaport, 2002; Di Donato et al., 2008) show that the primary charge separation starts either by electron transfer from P_A to B_A or from B_A to the primary acceptor $Pheo_A$, all bound within D1. The question is which of $P_A^+B_A^-$ and $B_A^+Pheo_A^-$ radical pairs is the initial product (Diner and Rappaport, 2002; Barber, 2006). The primary charge separation is then stabilized by fast electron transfer from $Pheo_A^-$ to a plastoquinone (Q_A) bound on the stromal side of the D2 protein. In a slower reaction, the electron is transferred to the secondary plastoquinone acceptor (Q_B) bound to D1. After a new light-induced charge separation reaction Q_B^- receives a second electron followed

by protonation forming plastoquinol (PQH_2). Reduced plastoquinone is displaced from the Q_B binding site to intermix with the plastoquinone pool of the thylakoid membrane and electrons are transferred to PSI via the cytochrome b_6f complex and eventually used to reduce $NADP^+$ to NADPH. The oxidized primary donor ($P680^+$) is an exceptionally strong oxidant and is thus able to extract electrons from water (Aro et al., 1993; Stewart and Brudvig, 1998; Nelson and Ben-Shem, 2004). $P680^+$ is reduced by a redox-active tyrosine residue of the D1 protein (Tyr_Z) which is re-reduced by an electron from a cluster of four Mn, one Ca^{2+} and Cl^- that constitutes the oxygen-evolving complex (OEC). During the oxidation of water the manganese cluster cycles between five different redox states (S_0-S_4) to release one molecule of oxygen. The side of Mn-cluster is capped by extrinsic proteins bound to the lumenal surface of the complex to protect it against other reductants than water (Barber, 2006). The OEC extrinsic proteins of higher plants and green algae are similar and consist of the PsbO, PsbP, PsbQ and PsbR subunits. With an exception of PsbR the cyanobacterial OEC contains the same proteins and two additional subunits designated PsbU and PsbV (cytochrome c-550) (Roose et al., 2007).

The character of redox reactions proceeding in PSII RC inevitable leads to the regular light-induced damage. Since the above described charge separation events occur mainly on the D1-side of the RC (the symmetrically related cofactors located on the D2-side (B_B and $Pheo_B$) are non-functional) (Barber, 2006) and also the Mn-Ca-Cl cluster is mostly coordinated by amino acid residues of D1 (Ferreira et al., 2004; Loll et al., 2005), the photoinhibitory damage is most probably located at this protein. The damaged D1 protein is then promptly degraded and re-synthesized, consequently D1 is the most rapidly turning-over protein within PSII (He and Vermaas, 1998; Barber, 1998). The respective rates of damage, degradation and synthesis of D1 are regulated by the light intensity (Alfonso et al., 1999; Inagaki et al., 2001). Recent investigations on the D1 replacement in cyanobacteria suggest that the selective degradation of D1 is dependent on availability of the newly synthesized D1 protein which seems to be co-translationally inserted into the complex after the old D1 protein is removed from the complex by the FtsH proteases (Nixon et al., 2005). The D1 protein is synthesized in the form of precursor (pD1) with an extension on its carboxyl terminus. The C-terminal extension is removed by a specific carboxyl-terminal processing protease. In *Synechocystis* this endoprotease designated as CtpA (Anbudurai et al., 1994) cleaves the 16 amino acid residues of the C-terminal extension in a two-step manner (Inagaki et al., 2001) with the first cleavage occurring after Ala352 (Komenda et al., 2007) and the final step located after Ala344 (Nixon et al., 1992).

1.4. Small PSII subunits

Besides D1, D2, CP43, CP47 and a set of extrinsic lumenal proteins, the PSII complex also contains a large number of small intrinsic subunits. These are highly conserved among oxygenic phototrophs and up to now at least 18 subunits putatively associated with the PSII complex have been identified. Most of them contain a single transmembrane helix and their function is largely unknown. They are assumed to be involved in stabilization, assembly and dimerization of the complex. The small proteins may facilitate fast conformational changes that the PSII complex needs to perform an optimal photosynthetic activity (for review see Shi and Schröder, 2004).

1.4.1. Cytochrome b-559

Cytochrome b-559 (cyt b-559) is a component of a highly purified PSII RC complex, which contains the D1 and D2 proteins and is still capable of primary charge separation (Cramer et al., 1986; Nanba and Satoh, 1987; Stewart and Brudvig, 1998). Cyt b-559 is comprised of two small polypeptides, the α (9 kDa) and β (4 kDa) subunits (Stewart and Brudvig, 1998), which bind one heme as a prosthetic group (Cramer et al., 1986). It is the only known membrane bound multisubunit cytochrome in which the two different subunits provide ligands to the heme iron (Pakrasi et al., 1991).

The α and β polypeptide subunits of cyt b-559 are encoded by the *psbE* and *psbF* genes, respectively, which are co-transcribed as part of the *psbEFLJ* operon in the unicellular cyanobacterium *Synechocystis* (McNamara et al., 1997). The nucleotide sequences of *psbE* and *psbF* are highly conserved among cyanobacteria and green plants, the homology between *Synechocystis* and spinach is 70% and 66% for *psbE* and *psbF*, respectively (Pakrasi et al., 1988). Nevertheless, the C-terminal part of the PsbE protein of *Synechocystis*, *Chlamydomonas* and *Cyanelle paradoxa* differ from that of higher plants (Vallon et al., 1989). The initial structural studies on cyt b-559 showed that it is located only in the regions of grana stacks (Rao et al., 1986), that the C-terminal domain of the α-subunit of cyt b-559 is

oriented towards the lumenal side and that the N-terminal domain faces the stromal side of the thylakoid membrane (Vallon et al., 1989; Tae and Cramer, 1994). Stoichiometry of cyt b-559 copies per PSII RC in photosynthetic organisms is still a matter of debate. One possibility is that different photosynthetic organisms contain different numbers of cyt b-559 copies. Another possibility is that under some conditions cyt b-559 is labile and can be lost from PSII during purification (Stewart and Brudvig, 1998).

Cyt b-559 possesses highly variable redox potential and according to this potential high-, low- and intermediate-potential forms can be distinguished in plant thylakoids (Arnon and Tang, 1988; Thompson et al., 1989; Poulson et al., 1995). Barber and De Las Rivas (1993) proposed an existence of the reversible switch between high and low potential forms of cyt b-559 which would allow protection against both the donor and acceptor side photoinhibition. The conversion between high potential and low potential form of cyt b-559 was found to be reversible in the light (Nedbal et al., 1992; Barber and De Las Rivas, 1993; Poulson et al., 1995) and the related change in the conformation of cyt b-559 itself was proposed to lead to a change in the entire structure of the PSII RC. This interconversion could affect the function of the acceptor side of PSII and act as a molecular switch to regulate the function of PSII (Mizusawa et al., 1999).

Insertion into the membrane and subsequent stabilization of cyt b-559 is one of the primary events which appear to be essential for the stable assembly of the functional PSII complexes (Pakrasi et al., 1988; Pakrasi et al., 1991; Shukla et al., 1992a; Cramer et al., 1993; Morais et al., 1998). In the absence of cyt b-559 due to deletion of the *psbE* and *psbF* genes, the D1 and the D2 proteins do not accumulate in the thylakoid membranes and there is no functional PSII RC in cyanobacterial cells (Pakrasi et al., 1989). Cyt b-559 has a heterodimeric structure so the loss of even one of two axial ligands is expected to destabilize the structure of the protein severely (Pakrasi et al., 1991). Site-directed mutants of the heme ligands cannot grow photoautotrophically (with an exception of one *Synechocystis* mutant) and are able to assemble no or only limited amount of stable PSII complexes in both *Synechocystis* (Hung et al., 2007) and *Chlamydomonas reinhardtii* (Morais et al., 1998). As in *Synechocystis*, the *Chlamydomonas* mutants lacking cyt b-559 subunits do not accumulate D1 and D2 in the membranes because of the reduced rate of their synthesis and/or their rapid degradation. The lumenally exposed region of cyt b-559 in *Synechocystis* is also essential for the correct function of PSII and its assembly (Shukla et al., 1992b) while the strains with the insertional mutations in the N-terminal domain of β-subunit contain the functional PSII complexes (Pakrasi et al., 1988; Tae and Cramer, 1994). Shukla et al. (1992a) proposed a

model for the assembly of the PSII complex: i) cyt b-559 is synthesized and inserted in the thylakoid membrane where it is stabilized by the co-ordination of its heme cofactor. D2 is synthesized and inserted into the membrane, where an association with cyt b-559 allows it to remain stably integrated; ii) D1 is incorporated in the RC only when the D2-cyt b-559 precomplex is present; iii) the remaining integral membrane proteins (CP47, CP43, possible small PSII polypeptides) are incorporated into the PSII complex only when the RC complex is stably assembled.

1.4.2. The PsbH protein

The PsbH protein belongs to the most frequently studied small subunits of PSII. The protein was first identified in pea chloroplast as a 9-kDa protein (Bennett, 1977) which undergoes phosphorylation (Mayes and Barber, 1990; Giardi et al., 1986) catalyzed by a light-dependent redox-regulated kinases (Bennett, 1991; Vainonen et al., 2005; Rochaix, 2007). The N-terminus of the plant PsbH contains two threonines, one in the position 2 and one in the position 4, and both can be phosphorylated (Michel and Bennett, 1987). PsbH is also present in cyanobacteria but in these organisms the N-terminal part, which is otherwise well conserved among the spinach, tobacco and wheat (Michel and Bennett, 1987), appears truncated (Mayes and Barber, 1990). Consequently, the molecular mass of the cyanobacterial protein is only 6 - 7 kDa (Race and Gounaris, 1993; Koike et al., 1989), the phosphorylation sites at the N-terminus are missing and the protein is not phosphorylated (Koike et al., 1989; Komenda et al., 2002). On the other hand, the C-terminal part of the protein (including the single membrane-spanning segment) remains highly conserved among cyanobacteria, algae and plants (Mayes and Barber, 1990).

A number of site-directed *psbH* mutants from various organisms were constructed in order to elucidate the function of the PsbH protein. In the *psbH*-less mutant of *Synechocystis* the absence of PsbH primarily effected PSII activity, but the strain was still able to grow photoautotrophically (Mayes et al., 1993). The absence of PsbH caused conformational changes of the Q_B binding niche that increased sensitivity of PSII to photoinduced damage and also affected the rate of D1 degradation (Mayes et al., 1993; Komenda and Barber, 1995). Moreover, the light-induced modification of the PSII proteins in the *psbH* deletion mutant provided evidence for an increased probability of the formation of reactive oxygen species within PSII lacking PsbH (Komenda et al., 2002; Komenda et al., 2003). These species

oxidized the D1 protein and this could be subsequently cross-linked with the α-subunit of cyt b-559 or fragmented (Komenda et al., 2003). A more detailed analysis showed a significantly lower level of PSII and an increased level of the unassembled D1 protein in the cells of the *psbH*-null mutant. The assembly of PSII was strongly retarded at the level of the RCII complex, at the stage when CP47 was supposed to be attached. The results implied that during PSII assembly, PsbH becomes attached to CP47 and only subsequently, the CP47-PsbH pair binds to the RC complex (Komenda, 2005; Komenda et al., 2005). The *psbH* disruptant of *Thermosynechococcus elongatus* could also grow photoautotrophically and was more sensitive to photoinhibition than the wild type (Iwai et al., 2006). The authors supposed that PsbH can be indirectly involved in the dimerization of PSII and this was in agreement with data on *Synechocystis* PsbH-null mutant which was also deficient in PSII dimers (Komenda et al., 2003). In contrast, the deletion of the *psbH* gene in *Chlamydomonas* resulted in the complete loss of photoautotrophic growth and the mutant was unable to assemble any functional PSII (Summer et al., 1997; O'Connor et al., 1998). The high level of conservation between the *psbH* gene sequence of higher plants and cyanobacteria was confirmed by a variety of experiments, in which the PsbH protein from *Synechocystis* was substituted by the PsbH from *Zea mays*. The eukaryotic protein was not only expressed but also assembled at the expected site of the cyanobacterial PSII (Chiaramonte et al., 1999) and could functionally replace the endogenous protein. Such a hybrid was able to grow photoautotrophically although it was more sensitive to light than the control wild type strain of *Synechocystis*.

Based on the mutant characterization several functions were assigned the PsbH protein: i) protection of PSII from photoinhibition (Race and Gounaris, 1993); ii) regulating the connection between the internal antennae (CP43, CP47) and the RC (Chiaramonte et al., 1999); iii) co-determining the structure of the Q_B site and optimizing the electron transfer rate between the two plastoquinone acceptors; iv) a proper structural conformation of D1 allowing the prompt removal of the damaged polypeptide and insertion of the newly synthesized one into the thylakoid membrane during the PSII repair cycle (Komenda et al., 2002; Bergantino et al., 2003); v) stabilization of PSII structure, namely the stable binding of CP47 to the D1-D2 heterodimer and dimerization of the core complex (Komenda et al., 2003).

In the first higher resolution structure of the cyanobacterial PSII obtained using crystals of PSII from *Thermosynechococcus elongatus* (Zouni et al., 2001) the identity of PsbH was tentatively assigned to one of three helixes located closely to the center of the PSII dimer. Due to insufficient resolution this assignment was mostly based on the previous studies showing function of PsbH in the stabilization of the PSII dimer and in the regulation of

electron transport between Q_A and Q_B. In the later, higher resolution structural models of PSII from *Thermosynechococcus elongatus* the PsbH protein has been placed between CP47 and the D2 protein on the periphery of the dimeric PSII core (Kamiya and Shen, 2003; Ferreira et al., 2004; Loll et al., 2005). This localization was supported by data from the cyanobacterium *Synechocystis* (the protein was shown to closely associate with CP47 and to stabilize its binding to D2) (Komenda et al., 2002) and from *Chlamydomonas* (the PsbH subunit was identified in the vicinity of the two transmembrane helices of cytochrome b-559 using a Ni^{2+}-NTA gold labeling of the His-tagged PsbH) (Büchel et al., 2001). The result was additionally confirmed by cross-linking of PsbH with PsbX, a small subunit which was in the latest PSII models located close to the α- and β-subunits of cyt b-559 and PsbH.

1.4.3. The PsbI protein

The simplest PSII unit capable of the primary charge separation consists of the D1-D2 heterodimer which can be isolated from plant thylakoids, mostly in a complex with the two subunits of cyt b-559 (Nanba and Satoh, 1987) and two small proteins encoded by the chloroplast *psbI* gene (Webber et al., 1989, Tomo et al., 1993) and the nuclear *psbW* gene (Ikeuchi et al., 1989). PsbI is a small, 4.8 kDa integral membrane protein which is evolutionary highly conserved (there is 71% amino acid identity between the *Arabidopsis* and *Synechocystis* proteins). Sequence analysis indicates that it contains only a single transmembrane helix with N-terminus on the stromal side of thylakoids (Shi and Schröder 2004). Cross-linking studies of the isolated RC complex from spinach indicated that the N-terminal domain of PsbI is in close contact with D2 and with the α-subunit of cyt b-559 on the stromal side of the thylakoid membrane (Tomo et al., 1993). Also the first more detailed crystal structure of isolated PSII from the cyanobacterium *Thermosynechococcus elongatus* assigned one α-helix in a position close to CP47 and D2 as the PsbI protein (Zouni et al., 2001). In contrast, the recent high resolution structures of the cyanobacterial PSII (Ferreira et al., 2004; Loll et al., 2005) showed PsbI in close contact with the first two helixes of D1 in the proximity of CP43.

The function of the PsbI protein in the assembly of the PSII RC complex remains largely unknown despite the construction and characterization of the specific *psbI*-null mutants of *Synechocystis*, *Chlamydomonas reinhardtii* and tobacco. The mutant of

Synechocystis lacking the *psbI* gene was able to grow photoautotrophically and its PSII oxygen evolution activity reached 70-75% of that found in the wild-type (Ikeuchi et al., 1995). In *Chlamydomonas reinhardtii* inactivation of the *psbI* gene decreased oxygen evolution activity and D1 content in the resulting mutant to 10-20% of the wild-type level (Künstner et al., 1995). The *psbI* deletion mutant of tobacco contained reduced amounts of dimeric PSII complexes and their supercomplexes with light harvesting antennae. The mutant also exhibited modified properties of the primary quinone acceptor Q_A, an increased sensitivity to high light and no phosphorylation of the RC proteins D1 and D2 (Schwenkert et al., 2006). In summary, the inactivation of *psbI* in all studied organisms led to the decrease in PSII photochemical activity and content but the underlying molecular basis for this decrease remained unknown.

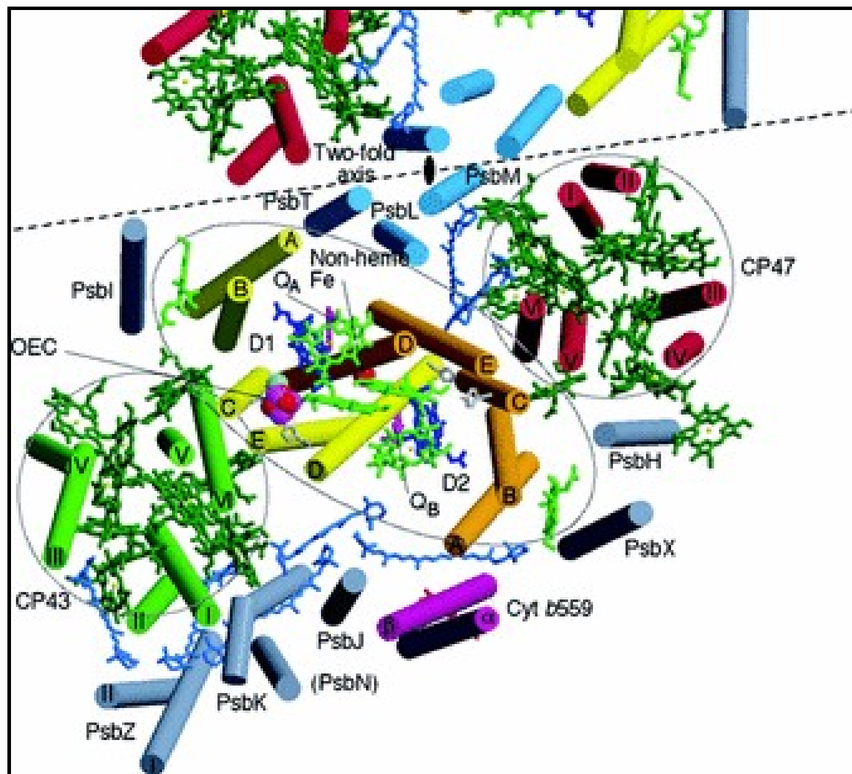

Fig. 2 Model of the PSII monomer along the membrane normal from the lumenal side showing organization of transmembrane α-helices and position of cofactors. From Ferreira et al., 2004.

1.4.4. The PsbW protein

As mentioned above, the nuclear-encoded PsbW protein was firstly identified in the isolated PSII RC complex from higher plants together with D1, D2, both subunits of cyt b-559 and PsbI (Ikeuchi et al., 1989; Irrgang et al., 1995; Lorković et al., 1995; Bishop et al., 2003). The protein is highly conserved among spinach, *Arabidopsis* and *Chlamydomonas* (Shi et al., 2000), its relative molecular weight is about 6 kDa and computer predictions indicate the presence of one transmembrane α-helix and orientation of the N-terminus to the thylakoid lumen (Irrgang et al., 1995; Lorković et al., 1995). In contrast to identification of the protein in the isolated PSII RC, Hiyama et al. (2000) detected the PsbW protein in both photosystems. However, the subsequent analysis of the *Chlamydomonas* membranes revealed the presence of PsbW exclusively in the purified PSII but not in PSI (Bishop et al., 2003).

As in the case of majority of other small PSII proteins the function of the PsbW protein in the PSII assembly is not clear. In the mutant of *Arabidopsis* lacking the PsbW protein no drastic changes in phenotype were observed in comparison to WT. However, the rate of oxygen evolution in the transgenic plants decreased by 50% due to decrease in the level of PSII proteins as evidenced by imunodetection of D1, D2, CP43, PsbO and PsbP proteins. In the absence of the PsbW protein the stability of the dimeric PSII was diminished and the PSII supercomplexes became undetectable (Shi et al., 2000). A later biochemical analysis of pea leaves showed that the PsbW protein is not associated with monomeric form of PSII but is apparently inserted directly into the dimeric PSII supercomplex with rapid kinetics (Thidholm et al., 2002). In contrast, a more recent study on assembly of thylakoid protein complexes (Rokka et al., 2005) revealed insertion of PsbW also into the PSII core monomer. This study also revealed that PsbW is synthesized only at low light intensities together with light harvesting Lhcb proteins and the protein was detected in the PSII-LHCII supercomplexes. The authors proposed the role for PsbW as a linker between LHCII and the PSII core complex and this idea was supported by localization of PsbW only in the granal PSII complex but not in the stromal ones.

PsbW seemed to be unique for higher plants but a gene for so called “PsbW” was detected also in the genomes of *Synechocystis*, *Synechococcus* 7002 and several types of algae (Shi and Schröder, 2004). The *Synechocystis* PsbW is a hydrophilic and significantly larger (10 - 13 kDa) protein than the plant PsbW. The sequence identity between the *Arabidopsis* PsbW protein and the *Synechocystis* PsbW is only 16%. The protein was identified in a highly active PSII preparation from *Synechocystis* and the authors designated it as the Psb28 protein

(Ikeuchi et al., 1995; Kashino et al., 2002). The Psb28 protein is present in the isolated PSII in a sub-stoichiometric amount but its higher accumulation was observed in PSII complexes from the phosphatidylglycerol-depleted cells of the *Synechocystis* mutant unable to synthesize this anionic lipid (Sakurai et al., 2007). The mutant of *Synechocystis* with interrupted *psb28* could grow autotrophically and only exhibited a slower growth in the absence of Ca^{2+} or Cl^- (Ikeuchi, unpublished data from the Kazusa Cyanobase). Recently, a soluble protein named PsbW-like protein has been identified in *Arabidopsis* and its C-terminal domain exhibited the 44,2% identity with this domain in the *Synechocystis* Psb28 (Shi and Schröder, 2004). The function of this plant protein is completely unknown.

2. THE AIMS

Small intrinsic subunits of PSII are found in all kinds of oxygenic photosynthetic organisms but mostly no details are known about their exact function. The aim of this thesis was to clarify the role of several selected small proteins in PSII. We chose four small PSII subunits:

1) cytochrome b-559 – we attempted to better define the role of cyt b-559 in the assembly of the cyanobacterial PSII using various mutant strains lacking one or more PSII core protein subunits
2) PsbH – the aim was to clarify the location of the PsbH subunit using Ni-affinity chromatography and NTA gold labeling
3) PsbI – the aim was to improve the available knowledge on function of the protein in the assembly and repair of the cyanobacterial PSII
4) Psb28 – the purpose was to confirm the presence of the protein in PSII, to localize it within PSII (the protein is absent in the recent PSII models - Ferreira et al., 2004; Loll et al., 2005), and to establish its importance for the cyanobacterial PSII.

For this purpose we used the cyanobacterium *Synechocystis* sp. PCC 6803, which is one of the most popular organisms for genetic and physiological studies of photosynthesis for several reasons: it is naturally transformable by exogenous DNA and possess efficient homologous recombination machinery, grows heterotrophically at the expense of glucose even in the absence of functional photosystems and its genome is completely known (Ikeuchi and Tabata, 2001).

3. RESULTS AND DISCUSSION

3.1. The formation of D2-cyt b-559 pre-complex as one of the starting points in the PSII assembly process

Results of Müller and Eichacker (1999) suggested that in plants etioplasts lacking photosynthetic apparatus formation of the D2-cyt b-559 pre-complex represents one of the first PSII assembly steps. Analysis of PSII protein subunits and their pre-complexes in the *Synechocystis* D1-less strain showed that similar pre-complex might also be formed in cyanobacteria. The shape of the band for the unassembled cyt b-559 extending to the position of the unassembled band of D2 differed from the more regular band found in the D2-less strain in which no interaction between cyt b-559 and D2 is possible (Paper I, Fig. 8D and E). The existence of D2-cyt b-559 pre-complex in the strain lacking D1 has been confirmed by co-immunoprecipitation of both cyt b-559 subunits and D2 using an antibody against the β subunit of cyt b-559 (PsbF) (Fig. 3). Moreover, 2D gel analysis of the ΔCYT strain lacking *psbEFLJ* operon confirmed that in the absence of cyt b-559 the synthesis of D2 is very limited and the D2 protein does not accumulate. On the other hand synthesis of the D1, CP43 and CP47 proteins does not seem to be affected by the absence of cyt b-559. However, these proteins accumulate in the membranes as unassembled subunits and they are not able to form any high-molecular weight complexes. Thus, we believe that formation of the D2-cyt b-559 pre-complex represents something like a crystallization point, from which PSII assembly in *Synechocystis* starts.

The cyt b-559 is present in all PSII assembly complexes including RC complexes but its significant amount remains unassembled even in the absence of D2. It is possible that the presence of surplus number of copies of the PsbE/PsbF heterodimer in comparison with D2 allows the prompt initiation of *de novo* assembly of PSII. This might be important under variable environmental conditions that require a fast response of the photosynthetic apparatus. This response should be faster if only the large subunits must be synthesized *de novo* while their stabilization factors are already present in the membrane. We cannot exclude either that the unassembled cyt b-559 represents a reservoir of membrane bound heme which can be quickly utilized whenever needed.

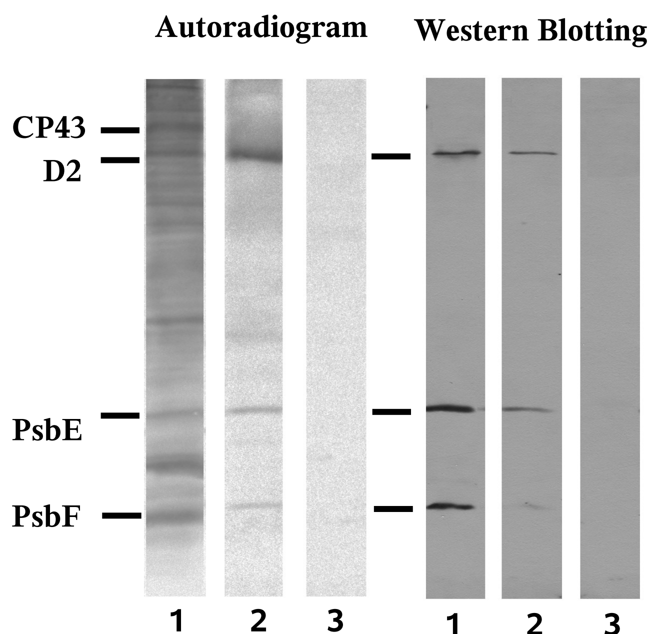

Fig. 3 Co-immunoprecipitation of D2 and cyt b-559 subunits from the ΔD1 strain. Co-immunoprecipitation of pulse-radiolabeled thylakoid membranes (5 μg of Chl) from strain lacking D1 was performed using the antibody against PsbF. The co-immunoprecipitates (2, 3) together with thylakoids from ΔD1 (1) were analyzed by SDS-PAGE, blotted onto PVDF membrane, autoradiographed (left panel, autoradiogram) and then probed with antibodies against D1, PsbE and PsbF proteins (right panel, Western blotting). 1 - thylakoid membranes; 2 - proteins co-immunoprecipitated using antibody against PsbF; 3 - proteins co-immunoprecipitated using a pre-serum.

3.2. PsbH acts as a stabilizing factor for the CP47 protein

The localization of the small PSII subunit PsbH within the cyanobacterial PSII core complex was recently matter of a controversy (Zouni et al., 2001; Kamiya and Shen, 2003). To clarify the location of the PsbH protein within PSII from *Synechocystis*, we decided to employ the Ni-NTA Nanogold methodology using the PSII complex isolated from a strain expressing the His-tagged PsbH protein (Paper II). The tagged PSII core complexes were isolated using Ni-affinity chromatography attached to the electron microscopy grid and subsequently labeled using Ni-NTA Nanogold according to Büchel et al. (2001). The labeled complexes were visualized by electron microscopy and the obtained particle images were aligned and treated with multivariate statistical analysis. The obtained averaged images showed that the PSII dimers preferentially attach with their stromal side to the carbon support film. Assuming the orientation of the N-terminus of PsbH to the stromal side of the

membranes this attachment seriously restricted the access of the gold particles to the tag. Therefore, only particles tilted in respect to the support carbon grid and only one of two positions on the PSII dimer could be labeled (Paper II, Fig. 5). Images of these gold labeled complexes showed the label in proximity of the transmembrane helices IV and V of the CP47 protein at the periphery of the dimer (Paper II, Fig. 6) and this was fully in agreement with the assignment of a helix in this PSII region to PsbH in 3D models of Kamiya and Shen (2003), Ferreira et al. (2004) and Loll et al. (2005). Interestingly, in *Chlamydomonas reinhardtii* using the same method PsbH was localized in slightly different position in the vicinity of the two cyt b-559 subunits (Büchel et al., 2001). Moreover, in contrast to the PSII complex from *Synechocystis* the *Chlamydomonas* complex was attached to the support film by the lumenal side. We believe that the observed differences between *Synechocystis* and *Chlamydomonas* are caused by the different length of the N-terminus of PsbH from both organisms. The N-terminus of the cyanobacterial protein is shorter by 19 amino acid residues in comparison with the protein from *Chlamydomonas* (Komenda et al., 2003). Therefore, it is possible that the N-terminus of the cyanobacterial PsbH protein is not long enough to reach cyt b-559 as the PsbH protein of *Chlamydomonas*.

When CP47 remains unassembled (for instance in the strains lacking D2 or D1) vast majority of PsbH remains associated with unassembled CP47 and in the absence of CP47 only negligible amount of PsbH is detected as a free protein on 2D gels (Komenda et al., 2005). This seems to be different from cyt b-559 which accumulates much higher extent as a free protein in the absence of its binding partner D2 (Paper I, Fig. 8). We assume that the expressions of PsbH and CP47 are interconnected. In agreement with this hypothesis we found that both synthesis and accumulation of the unassembled CP47 in the double deletion strain lacking both *psbEFLJ* and *psbH* (ΔCYT/ΔPsbH) is significantly lower in comparison with the single mutant ΔCYT (Fig. 4). Interestingly, the absence of PsbH also affected synthesis and accumulation of other chlorophyll binding proteins PsbC (CP43) and PsaA/B, the large subunits of PSI. The data indicate that synthesis of these chlorophyll-binding proteins might be co-regulated and deficiency in synthesis of one (in this case decreased synthesis of CP47 due to the missing PsbH) leads to deficiency in synthesis of the others. One possibility is that these proteins bind chlorophylls co-translationally and if CP47 is not fully complemented with chlorophyll, for example due to the absence of PsbH, it is subjected to proteolysis and the bound chlorophyll is also degraded. This process is assumed to occur frequently in the mutant and may lead to low availability of chlorophyll for synthesis of PsbC and PsaA/B. In contrast to these proteins, the synthesis of D1 was not affected in the double

mutant and this might reflect an existence of different mechanisms regulating synthesis of D1. This protein might bind chlorophyll post-translationally thus the synthesis of D1 would not be significantly affected by the chlorophyll deficit.

The unassembled CP47 usually exists as a double band on native gels (Komenda et al., 2005). Both bands were found to contain PsbH but the larger one contains in addition so called SCPs, small one-helix proteins homologous to transmembrane helixes of plant chlorophyll *a/b*-binding proteins (Promnares at al., 2006). The presence of PsbH was found to be essential for binding of SCPs to CP47 and single particle analysis of Nanogold-labeled PSII complexes with His tagged ScpD showed similar location of PsbH and ScpD in the PSII dimer. We believe that SCPs together with PsbH stabilize the newly synthesized CP47 before its assembly into PSII (Promnares et al., 2006).

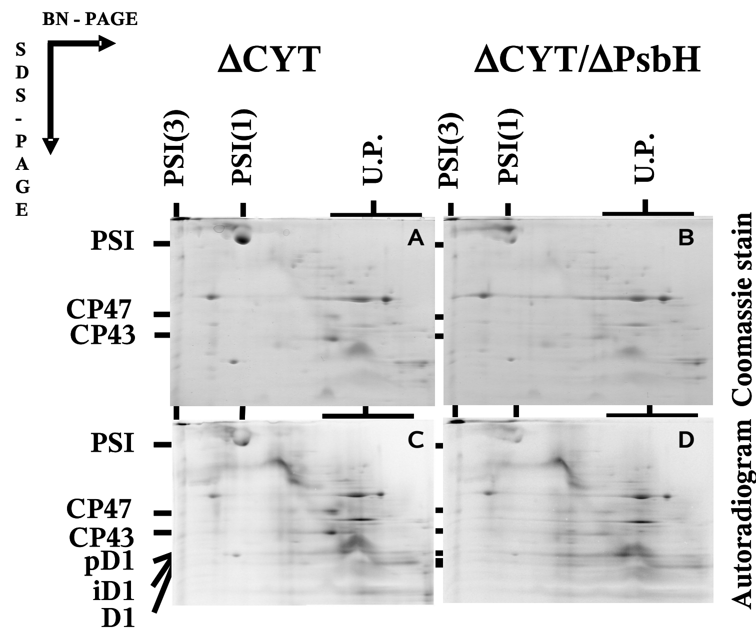

Fig. 4 Two-dimensional BN/SDS-PAGE analysis of ΔCYT and ΔCYT/ΔPsbH strains.
Cells of ΔCYT (A and C) and ΔCYT/ΔPsbH (B and D) were radiolabeled at 500 µmol photons m^{-2} s^{-1} with a mixture of [^{35}S]Met/Cys for 10 min (pulse). Labeled cells were used for isolation of thylakoids, which were analyzed in the first dimension by a native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12-20% polyacrylamide gel, stained with Coomasie blue (A, B) and autoradiographed (autoradiogram C, D). PSI (3) and (1) designates trimer and monomer of PSI, respectively; U.P. indicates unassembled proteins.

3.3. PsbI is an early assembly partner of D1 which also stabilizes binding of CP43 within PSII

The PsbI protein was originally discovered as the component of the PSII RC complex isolated from plant or cyanobacterial thylakoids by the detergent washing procedure which removes other subunits of the PSII core complex (Webber et al., 1989; Ikeuchi et al., 1989). Our results extend this knowledge by the finding that PsbI is also a component of the RC complex formed in the mutant of *Synechocystis* lacking CP47 during the PSII biogenesis process *in vivo*. In this *psbB*-null mutant, the PSII assembly cannot proceed beyond the stage of the RC complex since the next step, attachment of CP47, cannot occur (Paper I). Nevertheless, the RC complexes are formed in the absence of PsbI and we conclude that this protein is the component of the PSII RC complex but it is not critically required for its formation (Paper III, Fig. 3).

Our detailed analysis of the D1- and D2-less strains unable to form RC complex (Paper I) allowed us to look at assembly steps that even precede the formation of RC complex. As stated above, one of the early steps in biogenesis of the cyanobacterial PSII is the formation of D2-cyt b-559 pre-complex. Here we observed formation of another RC pre-complex consisting of D1 and PsbI. Support for existence of this specific pre-complex was based on its identification in several *Synechocystis* strains using 2D gel analysis (Paper III, Fig. 4A); on co-immunoprecipitation of PsbI and D1 from the D2-less strain using antibodies against both D1 and PsbI (Paper III, Fig. 4B); and on co-isolation of D1 with PsbI-His (Paper III, Fig. 4C). The absence of PsbI also led to the decrease in the amount of the unassembled D1 protein in WT and mutants lacking CP47 and D2. As the unassembled D1 seems to post-translationally associate with the D2-cyt b-559 pre-complex during *de novo* assembly of PSII we believe that PsbI play an important role in the stabilization of the D1 protein before it is utilized for assembly of new PSII complexes.

During the PSII repair D1 is assumed to be inserted co-translationally into RC47 (Zhang et al., 1999) and this insertion does not require PsbI (Paper III, Figs. 2 and 6). Interestingly, despite the binding partnership between PsbI and D1 in the functional PSII, PsbI is quite stable while D1 turns over very rapidly. Moreover, in the absence of PsbI the D1 turnover is even accelerated (Paper III, Fig. 6) and we believe that it is related to the destabilization of the CP43 binding to RC47 caused by the absence of PsbI (Paper III, Fig. 2) which most probably improves the access of D1 to the FtsH protease (for review see

Nixon et al., 2005). Here it is worth to note that similarly to subunits of cyt b-559 a significant fraction of PsbI occurs in the form of an unassembled protein in the thylakoid membranes of WT and even in the strain lacking D1. We again proposed that this fraction is important for the fast initiation of the *de novo* assembly of PSII whenever needed. Interestingly, the level of free pool of PsbI is controlled by the same FtsH protease (encoded by the *slr0228* gene) which also controls the selective D1 degradation during the PSII repair (Fig. 5). Therefore it is not clear why during the selective turnover of D1 the PsbI protein is not degraded.

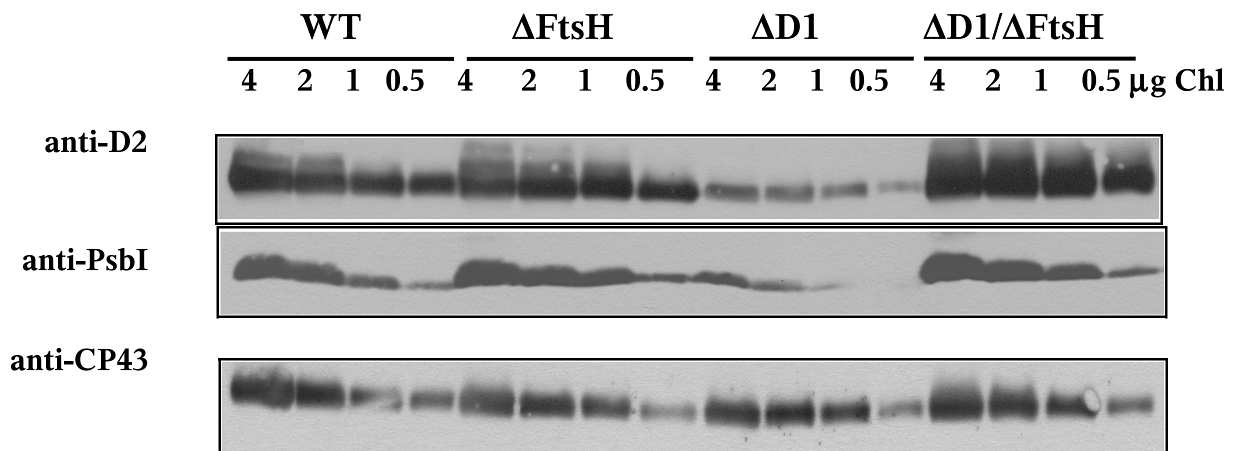

Fig. 5 Level of the unassembled PsbI protein is controlled by the protease FtsH (*slr0228*). Thylakoid proteins from cells of wild type (WT), mutant lacking FtsH (*slr0228*) protease (ΔFtsH), D1-less mutant (ΔD1) and the double mutant lacking both D1 and FtsH (ΔD1/ΔFtsH) were separated by denaturing SDS-PAGE. Proteins were detected using specific antibodies raised against the last 12 and 14 amino-acid residues of D2 and PsbI, respectively, and against isolated CP43 protein from *Synechocystis*. Correct protein loading was proven by blotting using specific antibody raised against the whole isolated CP43 protein from *Synechocystis*. The level of CP43 is not affected by FtsH (*slr0228*) (Komenda et al., 2006) and can be used as an internal standard for protein loading. 4, 2, 1 and 0.5 μg of chlorophyll were loaded onto the gel.

We also tested if the original PsbI can be functionally replaced by its version artificially extended with 6 histidines at the N-terminus. The obtained strain could be subsequently used for isolation of the PsbI-containing complexes. The *psbI* gene fused to His_6-encoding tag *(psbI-His)* was integrated into genome of the *Synechocystis* under strong promoter of the *psbA2* gene (one of two gene copies for the D1 protein). After the additional deletion of the original *psbI* gene the resulting strain was autotrophic and did not show any remarkable difference from WT as concerns the growth rate and photochemical properties of PSII. The presence of the His-tagged PsbI in the mutant thylakoids was proven using antibody

against PsbI that was reacting with the slightly larger protein than in WT (Fig. 6A). The PsbI-His protein was used for isolation of PSII core complex using Ni-affinity chromatography. The fraction of proteins bound to Ni^{2+} ions was eluted with imidazole and analyzed by 2D PAGE together with the PSII preparation from WT isolated using a combination of Cu-affinity and ionex chromatography (Komenda et al., 2002). During the native electrophoresis of the WT preparation the PSII core complex and its CP43-less subcomplex RC47 were separated (Fig. 6C). In contrast, the preparation from the PsbI-His/ΔPsbI strain contained four large PSII complexes, two cores and two subcores lacking CP43 (Fig. 6D). When we compared the protein composition of the monomeric core complexes RCC(1) from both preparations, each contained all four large subunits CP47, CP43, D2 and D1, both subunits of cyt b-559 and PsbH (Fig. 6B). As expected, in the mutant complex a new band corresponding to PsbI-His appeared while the band of the original PsbI disappeared. More interestingly, the additional two smaller complexes RCC(1)* and RC47* in the mutant differed from regular complexes by the absence of both cyt b-559 subunits indicating a certain structural relationship between PsbI and cyt b-559. The PsbI-His protein exhibited unusual behavior in comparison with PsbI. We were not able to detect PsbI-His using an antibody against 6xHis while using the antibody against PsbI protein was easily detected. Moreover, the antibody against PsbI detected the protein not only in the band with mobility corresponding to its size but there was at least one additional PsbI-related band that did not react with any antibody raised against various PSII subunits (PsbE, PsbF, D1 and D2). Therefore, we assume that the second band represented the dimeric form of PsbI. We assume that position of PsbI-His in the proximity of the RC results in oxidation of the His-tag. Such an oxidation was observed in plants expressing His-tagged proteins (Witte et al., 2004). The oxidized tag cannot react with the specific antibody against His-tag but can react with histidine residues or N-terminal amino groups of neighbor proteins (Lupínková et al., 2002). Such a reaction with the His-tag from another PsbI-His can result in formation of the observed PsbI-His dimer or the reaction with the amino group of cyt b-559 α subunit (PsbE) can cause detachment of cyt from PSII. Taking into account only one copy of PsbI per PSII monomer and the long distance between PsbI and PsbE, the only possibility is that PsbI from one complex interacts with PsbI or cyt b-559 from the different complex. This hypothesis can also explain the controversial results of the cross-linking study by Tomo et al. (1993). Oxidative modification of the His-tag is also supported by the behavior of the PSII complex containing PsbI-His on the nickel-affinity column. The PsbI-His-tagged complex is eluted already at 25 mM imidazole while the same PSII core complex with the His-tagged PsbH is eluted at 50 mM imidazole. Thus, PsbI-His

binds to the column much weaker than PsbH-His and this could be the reason why we were not successful in gold labeling of PsbI-His in the isolated PSII complex while this methodology was previously successful for PsbH-His (Paper II) or ScpD-His (Promnares et al., 2006).

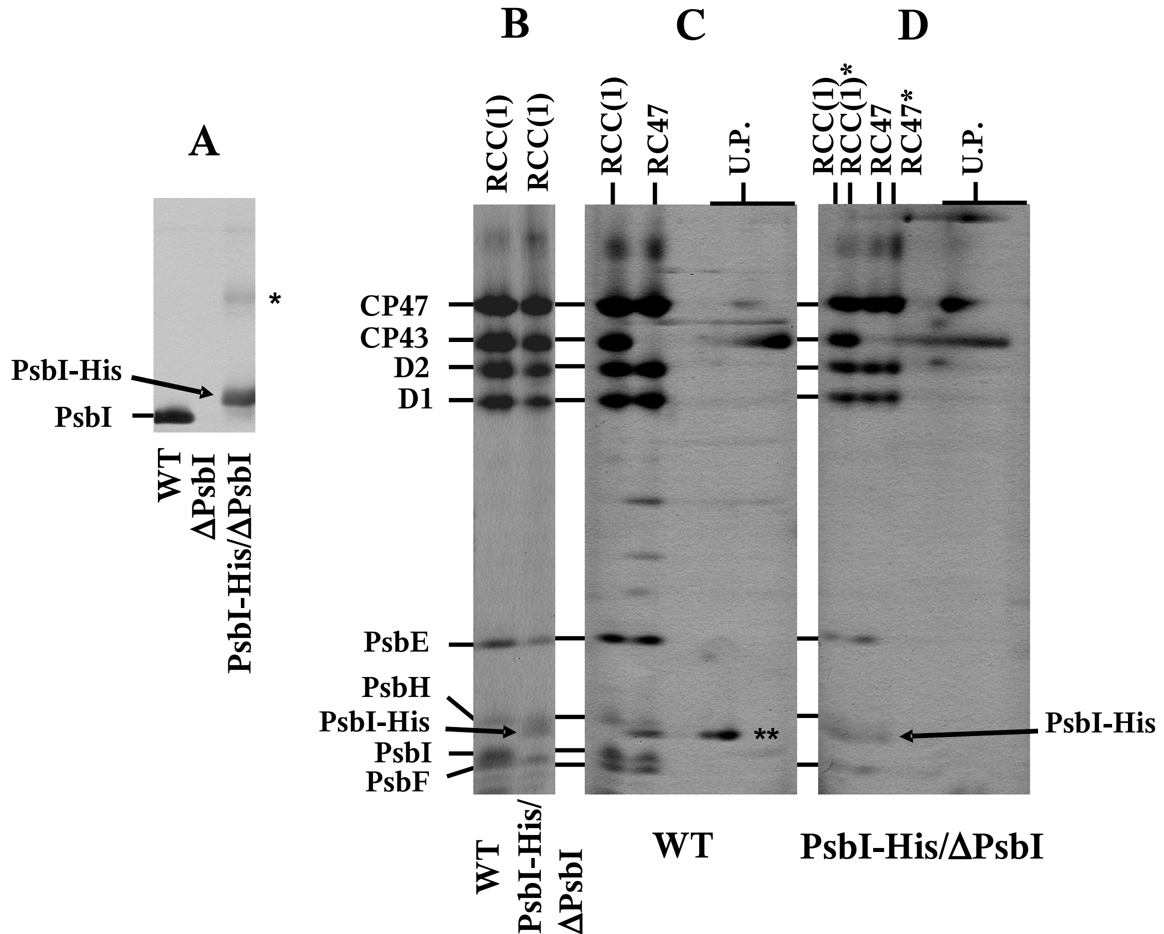

Fig. 6 Western blot and Coomassie stain of thylakoid membrane proteins from the *Synechocystis* WT strain, *psbI* deletion strain ΔPsbI and PsbI-His/ΔPsbI strain after their separation by SDS-PAGE or 2D BN/SDS-PAGE. (A): thylakoids from WT, ΔPsbI and PsbI-His/ΔPsbI strains were analyzed by SDS-PAGE, transferred onto nitrocellulose membrane and probed by antibody against PsbI protein, * designates putative dimer of PsbI-His; **(B):** PSII core complexes isolated from WT and PsbI-His/ΔPsbI strains analyzed by SDS-PAGE in the 12-20 % gradient polyacrylamide gel containing 7 M urea; **(C, D)**: PSII core complexes isolated from WT and PsbI-His/ΔPsbI strains separated by 2D BN/SDS-PAGE. ** designates the ATP synthase subunit c encoded by the *atpH* gene, RCC(1)* and RC47* are the two additional smaller complexes lacking both cyt b-559 subunits. Identity of small proteins PsbE, PsbF, PsbH and AtpH was confirmed by mass spectrometry.

3.4. Psb28 may facilitate utilization of chlorophyll released during D1 turnover for synthesis of CP47 and PSI

The Psb28 protein has been detected in a sub-stoichiometric amount in the purified PSII complex from *Synechocystis* by mass spectrometric analysis (Kashino et al., 2002). However, this protein is not a component of the highly active PSII complex from *Thermosynechococcus elongatus* which was used for crystallization and high resolution X ray structural analysis. Consequently, the protein is not seen in the 3D models of this complex (Ferreira et al., 2004 and Loll et al., 2005) and remains to be clarified if it is a true PSII component, a transient assembly factor or an impurity. For this purpose we screened various types of PSII complexes and PSII assembly intermediate for the presence of Psb28. We found that it binds preferentially to RC47 and only small amount was occasionally found also in the monomeric RCC(1) complex (Paper IV, Fig. 2). The presence of Psb28 in RC47 was especially obvious in certain mutants containing a large amount of RC47, namely the mutant PAL lacking all phycobiliproteins and having an unusually high PSII/PSI ratio (Aljani and Vernotte, 1998), and in the site directed mutant D1-Asn359His (Kuviková et al., 2005) (Fig. 7).

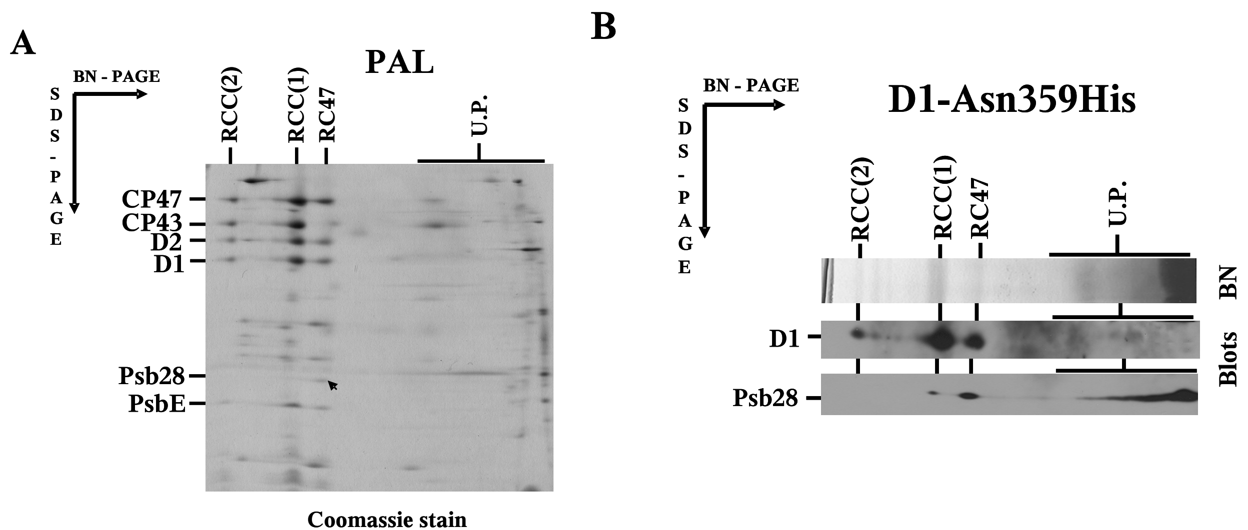

Fig. 7 Identification of Psb28 in the PSII complexes of the phycobilisome-less strain PAL (A) and the site-directed mutant D1-Asn359His (B). Membrane proteins of both strains were separated by 2D BN/SDS-PAGE. PAL proteins in the gel were stained by Coomassie blue, proteins of the D1-Asn359His mutant were blotted onto PVDF and immunodetected by antibodies against the D1 protein and Psb28. The arrow indicates the stained Psb28 protein which was identified by mass spectrometry.

We were also able to isolate the RC47 complex containing a nearly stoichiometric amount of Psb28 from the strain lacking CP43. We also isolated RC47 complex using strain expressing His-tagged Psb28 protein. We attempted to label the isolated RC47 using Ni-NTA Nanogold and to localize Psb28 within the complex. However, this was not successful due to the small size of RC47 which did not allow the reliable alignment of the obtained particle images.

The Psb28 mutant exhibited an apparently slower autotrophic growth than the WT strain and its relative PSII/PSI ratio was increased as suggested by 77 K chlorophyll fluorescence spectra and protein analyses. On the other hand, the deletion of *psb28* gene did not affect any functional properties of PSII what was in agreement with the transient character of its binding to the assembly complex RC47, and with its absence in the functional PSII dimer. The radioactive labeling of proteins showed that the synthesis of CP47 and PSI proteins PsaA/B was slowed down due to *psb28* deletion. These data indicated that Psb28 may control availability of chlorophyll for synthesis of various chlorophyll-binding proteins. We proposed three possible scenarios for the Psb28 function: i) binding of Psb28 to RC47 limits the D1 replacement process which is considered as the potentially most intensive consumer of chlorophyll; consequently there is more chlorophyll available for synthesis of other chlorophyll-binding proteins; ii) binding of Psb28 does not affects the rate of D1 replacement but improves reutilization of chlorophyll during the replacement process leading to higher chlorophyll availability for synthesis of other chlorophyll-binding proteins; and iii) Psb28 facilitates re-utilization of chlorophyll released during the D1 turnover for synthesis of other chlorophyll proteins. The assessment of the D1 turnover and PSII repair in the Psb28-less strain confirmed an accelerated turnover of D1 and showed higher efficiency of the PSII repair pointing out to the first possibility. However, in the Psb28-null strain we detected an increased excretion of a degradation product of chlorophyll into the growth medium indicating that the available chlorophyll is degraded instead of being utilized for the synthesis of other chlorophyll proteins. Thus, we propose that Psb28 facilitates utilization of a fraction of chlorophyll released during the D1 degradation for synthesis of other chlorophyll-proteins.

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5. ENCLOSED PUBLICATIONS

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Accumulation of the D2 Protein Is a Key Regulatory Step for Assembly of the Photosystem II Reaction Center Complex in *Synechocystis* PCC 6803*

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Accumulation of monomer and dimer photosystem (PS) II reaction center core complexes has been analyzed by two-dimensional Blue-native/SDS-PAGE in *Synechocystis* PCC 6803 wild type and in mutant strains lacking genes *psb*A, *psb*B, *psb*C, *psb*DIC/DII, or the *psb*EFLJ operon. *In vivo* pulse-chase radiolabeling experiments revealed that mutant cells assembled PSII precomplexes only. In Δ*psb*C and Δ*psb*B, assembly of reaction center cores lacking CP43 and reaction center complexes was detected, respectively. In Δ*psb*A, protein subunits CP43, CP47, D2, and cytochrome b_{559} were synthesized, but proteins did not assemble. Similarly, in Δ*psb*D/C lacking D2, and CP43, the *de novo* synthesized proteins D1, CP47, and cytochrome b_{559} did not form any mutual complexes, indicating that assembly of the reaction center complex is a prerequisite for assembly with core subunits CP47 and CP43. Finally, although CP43 and CP47 accumulated in Δ*psb*EFLJ, D2 was neither expressed nor accumulated. We, furthermore, show that the amount of D2 is high in the strain lacking D1, whereas the amount of D1 is low in the strain lacking D2. We conclude that expression of the *psb*EFLJ operon is a prerequisite for D2 accumulation that is the key regulatory step for D1 accumulation and consecutive assembly of the PSII reaction center complex.

The photosystem II (PSII)[1] reaction center core (RCC) complex of higher plants, algae, and cyanobacteria can be subdivided into a heterodimer containing D1 and D2, the antenna proteins CP47 and CP43, and a large number of low molecular weight integral membrane proteins including the α and β subunits of cytochrome b_{559} (α and β cytochrome b_{559}) (1–3). The heterodimer and antenna proteins are essential for binding the prosthetic groups needed for energy and electron transfer (4) as well as for binding the multitude of plastid-encoded small subunits, *e.g.* Psb-H, -J, -K, -L, and Psb-T, which affect the function of PSII (5–8). Furthermore, plastid-encoded subunit psbZ has been shown to be required for attachment of CP26 during assembly of PSII-LHC supercomplexes, whereas the nucleus-encoded subunit psbW was demonstrated to be required for RCC dimer formation (9–11). The role of plastid-encoded subunits Psb-I, -M, and -N and the nucleus-encoded small subunits Psb-R, and X remains unclear. A striking feature of PSII is the fast turnover of the D1 protein that is believed to be required for PSII repair and restoration of its photochemical activity after photoinactivation (12, 13). Maintaining PSII function may require selective replacement of this central PSII subunit including an efficient apparatus to recognize inactive complexes, and remove damaged and insert a new D1 copy (5, 14, 15). Zhang *et al.* (16) suggested that D1 replacement in higher plants may occur cotranslationally in a PSII subcomplex consisting of at least D2 and CP47, hence eliminating the need for complete disassembly and *de novo* assembly from PSII subunits.

Cyanobacteria are an excellent model organism to study PSII assembly. The strain used most frequently is *Synechocystis* PCC 6803 because it is easily transformable, grows photoheterotrophically, and its genome has been completely sequenced (17). Mutants deficient in photosynthetic activity are easily isolated and defects caused by the mutation can be characterized well by biochemical and genetic means. Many mutants are available that still accumulate PSII complexes despite their functional impairment (18–20), whereas in eukaryotic algae or higher plants, similar mutations often lead to a complete disappearance of the complex making it difficult to investigate the residual assembly capability of the system (18, 21).

In the green alga *Chlamydomonas reinhardtii*, de Vitry *et al.* (22) described the importance of certain PSII subunits for the accumulation of PSII. Using several mutants they concluded that the D2 protein is necessary for synthesis of other large subunits like CP47 and D1, whereas D1 synthesis was not required for synthesis of D2 and CP47. In the absence of CP47, D1 and D2 were expressed without protein accumulation, and CP43 associated with PSII in the later stage of the assembly process. Analysis of *Synechocystis* mutants lacking PSII subunits (23–27) largely confirmed results from *Chlamydomonas*. In the absence of CP43, subunits CP47, D1, and D2 accumulated. In the absence of CP47, subunit CP43 accumulated, whereas D1 and D2 proteins became detectable by radiolabeling only. In the absence of D2, only small amounts of CP47, but no D1 were reported, and in the mutant lacking the D1 protein (28), CP43 still accumulated and small amounts of D2 and CP47 were also detected. Although, these data provided infor-

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[1] The abbreviations used are: PSI and PSII, photosystem I and photosystem II; BN, blue-native; Chl, chlorophyll; DM, dodecylmaltoside; Rubisco, ribulose-1,5-bisphosphate carboxylase; WT, wild type; RC, reaction center; RCC, reaction center core; iD1, intermediate D1.

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TABLE I
Synechocystis PCC 6803 mutants and PSII activity

Strains	PSII protein missing	PSII oxygen evolution	PSII	Ref.
			% of WT	
WT		+	100	60
Δ*psb*C	CP43	−	~20	23
Δ*psb*B	CP47	−	<10	24
Δ*psb*B/C	CP47, CP43	−	<10	This study
Δ*psb*A	D1	−	0	28
Δ*psb*D/C	D2, CP43	−	0	31
Δ*psb*EFLJ	α,β cytochrome b_{559}, PsbL, PsbJ	−	0	29, 32

mation with respect to subunit composition, the assembly of PSII subunits remained enigmatic.

A protein shown to be of importance for the initiation of PSII accumulation in higher plants and cyanobacteria is cytochrome b_{559}. In cyanobacteria, no accumulation of PSII was detected in the absence of one or both subunits (29, 30). Furthermore, no D2 or D1 was found, but PSII subunits CP47 and CP43 did accumulate (29, 31). Cytochrome subunits α and β are encoded by genes *psb*E and *psb*F, respectively (32). In plants and cyanobacteria they are part of the *psb*EFLJ operon and are cotranscribed with PSII subunits L and J. In *Chlamydomonas*, where *psb*E is transcribed separately from other PSII genes, D1, D2, and CP47 did not accumulate when *psb*E was deleted confirming the importance of cytochrome b_{559} for accumulation of PSII (33). Furthermore, recent characterization of *psb*J and *psb*L deletion mutants in plants confirmed that these proteins do not significantly affect accumulation of PSII RCC monomers but may be involved in proper assembly of the oxygen evolving apparatus in cyanobacteria and higher plant chloroplast (34–38).

Protein complexes solubilized from thylakoid membranes can be separated by sucrose density gradient centrifugation (39, 40). Identification of the subunit composition throughout the gradient is then performed by SDS-PAGE and gel blot analysis. This method is highly useful to study the assembly of photosystem protein complexes when combined with analysis of deletion mutants lacking expression of single complex subunits. This method has been intensively used to study assembly of PSII in isolated chloroplasts and thylakoids from spinach and was later complemented by nondenaturing Deriphat-PAGE and IEF (41). Results indicated that PSII assembly requires a sequential attachment of cytochrome b_{559}, psbI, CP47, and CP43 onto an initially formed D1–D2 heterodimer (6). However, because of limited resolution capacity of the sucrose gradient an alternative technical approach was used here. Protein complexes and corresponding protein subunits of thylakoid membranes were resolved by an improved two-dimensional separation technique based on Blue-native electrophoresis (42–44). Assembly of protein subunits was followed by pulse-chase radiolabeling of wild type and mutant *Synechocystis* PCC 6803 cells. *In vivo* pulse-chase radiolabeling experiments using mutant Δ*psb*A, Δ*psb*B, Δ*psb*C, Δ*psb*D, and Δ*psb*EFLJ strains indicated that only PSII precomplexes were assembled in the mutants. Data corroborated a sequential assembly of RCC monomers from reaction center (RC) and show that expression of the *psb*EFLJ operon is a prerequisite for D2 accumulation, which is the key regulatory step for stabilization of the newly synthesized D1 and the consecutive assembly of the PSII RC complex.

MATERIALS AND METHODS

Strains and Culture Conditions—*Synechocystis* PCC 6803 strains used for the study are described in Table I. Strains were grown in BG-11 medium supplemented with 5 mM glucose. In addition, plate medium contained 1.5% agar and 0.3% sodium thiosulfate (32). Liquid cultures of 50–100 ml were gently stirred in conical flasks and irradiated with 80–100 μE m^{-2} s^{-1} of white light at 29–30 °C.

Pulse-Chase Labeling of Cyanobacterial Cells—Cells (75 μg of Chl) in the late-exponential growth phase (2–4 μg Chl ml^{-1}) were harvested by centrifugation, washed, and resuspended in fresh BG11 to a final volume of 250 μl. The cell suspension was shaken in 2-ml Eppendorf tubes at 60 μE m^{-2} s^{-1} for 1 h at 30 °C. Then 12.5 μl of [^{35}S]methionine (>1000 Ci/mmol, Amersham Biosciences) was added (final activity of 500 μCi ml^{-1}) and illumination was continued for another 30 min. After this period, cells were immediately frozen in liquid nitrogen and used for preparation of thylakoids.

Preparation of Thylakoid Membranes—Thylakoid membranes were prepared as described (45). Briefly, cells were broken by vortexing with glass beads and separated from unbroken cells, cell debris, and soluble proteins by differential centrifugation.

Protein Analysis and Autoradiography—Prior to analysis, thylakoids (10 μg of Chl) were sedimented and then resuspended in 60 μl of ACA buffer (42, 43) to which 6 μl of 10% dodecylmaltoside was added. Thylakoids were incubated for 10 min on ice and then quickly centrifuged to remove unsolubilized material. Supernatant was mixed with 5 μl of loading buffer and applied on a 6–12% Blue-native polyacrylamide gel (BN-PAGE). When the buffer front had reached about ⅓ of the resolving gel, the upper buffer containing Coomassie Blue G-250 was replaced by the same buffer without the stain and separation was continued until the front had reached the end of the gel. Individual lanes containing the protein complexes were cut out and complexes were denatured within the gel lane by incubation for 20 min in solubilization buffer containing 33 mM Na_2CO_3, 2% SDS, and 0.66% 2-mercaptoethanol. Lanes were placed on top of a 12.5% polyacrylamide gel containing 4 M urea and protein subunits loaded with SDS were released from the gel and separated by SDS-PAGE along the electrical field gradient according to their molecular mass.

To separate D2 and the three D1 forms, a 12–20% linear gradient polyacrylamide gel containing 7 M urea was used. First dimension BN-PAGE lanes were fixed with 0.5% agarose in the upper electrophoretic buffer, and SDS-PAGE (46) was run overnight using the Ettan Dalt II system (Amersham Biosciences, 12% gel) or Protean xi cell (Bio-Rad, 12–20% gradient gel). Gels were stained with Coomassie Blue R, destained, dried, and then exposed to a phosphorimager plate. Alternatively, proteins from the unstained gels were electroblotted onto polyvinylidene difluoride membrane and the membrane was used for visualization of radiolabeled D1, D2, and CP43 proteins and for identification of proteins by gel blot analysis using specific antibodies raised against the N- and C-terminal parts of D1, D2, and CP47, the complete protein sequence of CP43, and the α subunit of cytochrome b_{559} protein. Specifically, antibodies were raised against (i) residues 58–86 of D1 from spinach; (ii) the last 16 residues of *Synechocystis* D1 precursor by Nixon (47); (iii) the last 12 residues of plant D2 by Nixon (47); (iv) residues 380–394 of barley CP47 by us; and the whole isolated CP43 (v) and α cytochrome b_{559} protein (vi) from *Synechocystis*. For estimation of the D1 and D2 protein content, the thylakoids containing 4, 2, 1, and 0.5 μg of chlorophyll were analyzed by standard SDS-PAGE on 12–20% linear gradient polyacrylamide gel containing 7 M urea. Proteins were electroblotted onto polyvinylidene difluoride membrane and immunodecorated by specific antibodies. In a molecular mass window from 50 to 700 kDa for BN-PAGE and 25–55 kDa for SDS-PAGE, Coomassie-stained proteins that remained undetected by gel blot analysis were identified by *de novo* sequencing using ESI-MS/MS on a Q-TOF mass spectrophotometer (Micromass, Manchester, UK).

Analysis of Assembly After Two-dimensional Native/SDS-PAGE—A two-dimensional analysis of the assembly process is based on the finding that an assembly step results in binding of a radiolabeled protein subunit to an assembly partner that can be detected during pulse-chase

radiolabeling as molecular mass shift of the radiolabeled protein by the first dimension of BN-PAGE. In the second dimension SDS-PAGE, the molecular mass shift caused by assembly of the protein subunit is indicated by a horizontal shift of radiolabel from the low to the high molecular mass region of the two-dimensional gel. If accumulation of radiolabel in a PSII subunit protein is found at a specific *x/y* position in wild type (WT) cells but cannot be detected in the mutant cell, we conclude that the deleted structural subunit is required for this particular assembly step. In the mutant, lacking expression of a particular structural subunit, an intermediate assembly state awaiting the scheduled subunit, may then accumulate. If the molecular mass of PSII assembly intermediates is compared between mutants, the sequence of single assembly steps is read from the lowest to the highest molecular mass subcomplex.

Chlorophyll Content—For measurement of chlorophyll concentrations, cells were sedimented by centrifugation and extracted with 100% methanol. The concentration of chlorophyll was calculated from the absorbance values of the extract at 666 and 720 nm (48).

RESULTS

Assembly of PSII Is Monitored by Two-dimensional BN/SDS-PAGE Separation of Thylakoid Membrane Protein Complexes—Assembly of PSII was studied in wild type and mutant *Synechocystis* 6803 cells illuminated in the presence of [^{35}S]Met. Radiolabeling was necessary to reach the highest level of sensitivity for detection of PSII assembly intermediates *in vivo*. Synthesis of PSII subunits D2, D1, CP47, and CP43 and their assembly was monitored by radiolabel accumulation in protein complexes, whereas the steady state level of protein subunits in complexes was monitored by Coomassie staining or by Western blotting after two-dimensional native/SDS-PAGE. For two-dimensional analysis, cells were broken and thylakoids were isolated. Thylakoids were solubilized and protein complexes were separated according to molecular mass by BN-PAGE (Fig. 1*A*).

We began by characterizing all proteins detected by Coomassie staining of thylakoids from WT cells. Using gel blot analysis the subunits of five protein complexes could be identified (Fig. 1*A*, Table II). At about 550 kDa, the α-, β-, and γ-subunits marked the position of the ATPase complex, and at about 400 kDa, the LSU subunit indicated the position of ribulose-1,5-bisphosphate carboxylase (Rubisco). Protein subunits of PSII, CP47, CP43, D2, and D1 accumulated in two complexes at about 300 and 600 kDa in WT cells, corresponding to monomeric and dimeric reaction center core complexes RCC1 and RCC2, respectively. Finally, at a molecular mass of about 70–90 kDa, unassembled CP47 and CP43 were detected. Because it cannot be determined from the BN-PAGE analysis whether these subunits are precursor protein complexes required for assembly of PSII or are released from native complexes during solubilization of the thylakoid membrane or BN-PAGE, we investigated the *de novo* assembly of PSII by pulse and pulse-chase treatment of *Synechocystis* cells. Furthermore, mass spectrometry was employed to identify proteins in the BN/SDS-PAGE window stained with Coomassie but not identified as PSII subunits by gel blot analysis (Table II).

Pulse radiolabeling of *Synechocystis* cells for 2.5 min with [^{35}S]Met readily identified PSII subunits CP47, CP43, D2, and D1 because all radiolabeled subunits were located precisely at the molecular mass position of protein subunits identified as RCC1 subunits by two-dimensional gel blot analysis (Fig. 1*B*, *RCC1*). Interestingly, one protein was identified in RCC1 with an intermediary molecular mass between a high molecular mass form identified as precursor D1 (pD1) and the mature D1 (D1) in the low molecular mass edge of the BN gel (Fig. 1, *B* and *E*). Because all three forms were identified by gel blot analysis with a C-terminal anti-D1 antibody, we termed this unprocessed D1 form intermediate D1 (iD1) (Fig. 1*E*, also see Fig. 3 and Refs. 49 and 50). The iD1 form was also found in a reaction

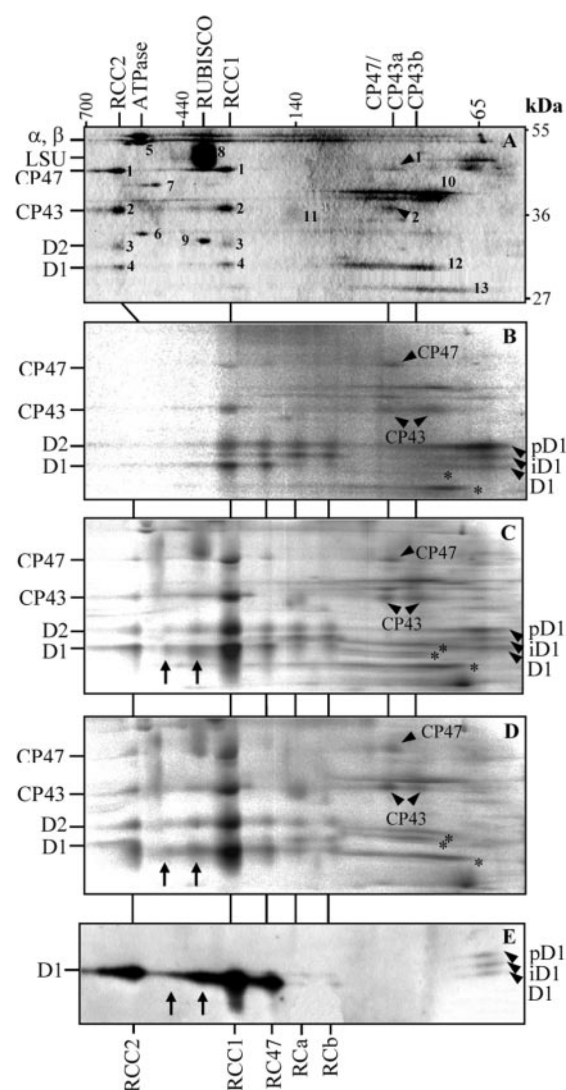

FIG. 1. **Coomassie stain, autoradiogram, and Western blot of thylakoid membrane proteins from the wild-type of *Synechocystis* PCC 6803 after separation of proteins by two-dimensional BN/SDS-PAGE.** A molecular mass window from 50 to 700 kDa for BN-PAGE and from 25 to 55 kDa for SDS-PAGE is presented to analyze the molecular mass increase of PSII complexes during assembly of protein subunits. Thylakoid membrane proteins from *Synechocystis* cells were radiolabeled with a mixture of [^{35}S]methionine and cysteine. Protein was separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in a 12.5% polyacrylamide gel and stained with Coomassie (*A*), pulse radiolabeled for 2.5 min (*B*), for 30 min (*C*), or pulse radiolabeled for 30 min and radiolabel chased for 30 min (*D*), or blotted onto polyvinylidene difluoride membrane and immunodecorated using anti-D1 antibody (*E*). Proteins were identified by gel blot analysis and mass spectrometry and were labeled as: α,β, subunits of CF1 part of ATP synthase; *LSU*, large subunit of Rubisco; *CP47*, *CP43*, *D2*, *pD1*, *iD1*, *D1*, reaction center core proteins of PSII; *RCC2* and *RCC1*, dimeric and monomeric PSII core complexes; *RC47*, PSII core complex lacking CP43; *RCa* and *RCb*, reaction center complexes containing D2, iD1, and D1. PSII complexes of unclear composition between RCC1 and RCC2 are designated by *vertical arrows*, unassembled CP47, CP43, and D1 forms are designated by *oblique arrows*. Non-PSII proteins in the region around the D1 protein are designated by *asterisks*. The *number* in *A* refers to Table II.

center core subcomplex with a molecular mass of 220 kDa termed RC47, which contained CP47, but no CP43 (Fig. 1*B*). In addition, two complexes with a molecular mass of 110 and 140 kDa were identified which assembled no mature D1 form (Fig. 1*B*). Because of the radiolabeling of iD1 and identification of D2 in these complexes (Fig. 3*D*), we called these complexes reaction center complexes and labeled them with a and b, according

TABLE II
Identification of proteins separated by two-dimensional BN/SDS-PAGE

Spot	Antibody detection	MS analysis
1	slr0906, CP47	
2	sll0851, CP43	
3	Sll0849, slr0927, D2	
4	Slr1311, sll1867, D1	
5		slr1326 and slr1329, α and β subunits of ATPase
6		slr1327, γ subunit of ATPase
7		slr0261, NAD oxidoreductase subunit H
8		slr0009, large subunit of Rubisco
9		Sll1363, ketol-acid reductoisomerase
10		sll0680, putative phosphate-binding periplasmic protein
11		sll0108, putative ammonium transporter
12		slr0447, negative aliphatic amidase regulator
13		sll1694, general secretion pathway protein G

to their molecular mass (140 kDa, RCa, and 110 kDa, RCb). In both RCC1 and RC47, D1 radiolabeling was increased relative to D2, pD1, and iD1, whereas pD1 was principally in its free form. Because D2 and D1 contain about equal numbers of Met residues, these results indicate a selective D1 turnover in both complexes.

When we extended the pulse labeling time, radiolabeled D1, D2, CP43, and CP47 proteins accumulated primarily in RCC1 (Fig. 1*C*), whereas only a minority of the radiolabel accumulated in the dimeric RCC (Fig. 1*A*, RCC2). Also, two RCC-like complexes were detected at a molecular mass intermediary between monomeric and dimeric RCC (Fig. 1, *C–E*, *vertical arrows*). Also, in RCC1 radiolabel intensity decreased in CP47 and increased in D1 relative to CP43/D2 (Fig. 1, *C* and *D*). Because about the same number of Met residues are present in CP47/CP43/D2/D1 we concluded that turnover rates of proteins and accumulation into the RCC1 complex are regulated independently. By chasing the radiolabel with unlabeled Met, pD1 labeling in the free protein fraction decreased, whereas iD1 labeling disappeared in the RCC1 complex and decreased in the RC47 complex. However, iD1 labeling was maintained in the RCa and RCb complexes and some D1 accumulated in both complexes (Fig. 1*C*, *iD1* and *D1*). Furthermore, no pD1 could be detected in any of the PSII complexes by gel blot analysis because iD1 was localized to the RC47, RCa, and RCb complexes (Fig. 1*E*). We therefore concluded that fast processing of pD1 paralleled formation of the reaction center, whereas slow processing of iD1 paralleled formation of RC47. Also, stable accumulation of D1 was only detected after assembly of the RC47 complex, indicating that binding of CP47 increased D1 stability (Fig. 1*E*). Hence, we concluded that reaction center complexes RCa and RCb are assembly transition intermediates for RC47 formation.

During pulse labeling, radiolabel accumulation in CP47 was initially equally distributed between the 70- and 90-kDa region of the two-dimensional gel (CP47) and the RCC1 complex, whereas less label was found in the RC47 complex (Fig. 1*B*, *CP47*). Radiolabeled CP43 appeared in RCC1 and in two small complexes termed CP43a and CP43b, according to their decreasing molecular mass (Fig. 1*B*, *CP43*). During the chase treatment, radiolabel in CP43b preferentially shifted into CP43a and into the RCC1 complex indicating that CP43a is the direct precursor protein complex for assembly of RCC1. Furthermore, chase treatment of WT cells enabled us to clearly distinguish between D1 forms and non-PSII proteins (Fig. 1, *C* and *D*, *asterisks*).

A Synechocystis Mutant Lacking psbC Is Characterized by Accumulation of RC47—To investigate the role of CP43 for *de novo* assembly and dimerization of RCC, we separated protein complexes and corresponding protein subunits from a *Synechocystis psb*C deletion strain. In the mutant, abundance of ATP synthase and Rubisco was found comparable with WT cells and these complexes accumulated to a level detectable by Coomassie staining (Fig. 2, *A* and *B*). CP47 as well as D2 and D1 were detected by Coomassie staining at the molecular mass of the RC47 complex. However, although CP47 accumulated in the RC47 complex, radiolabel intensity revealed an equal amount of CP47 in the 70–90-kDa complex.

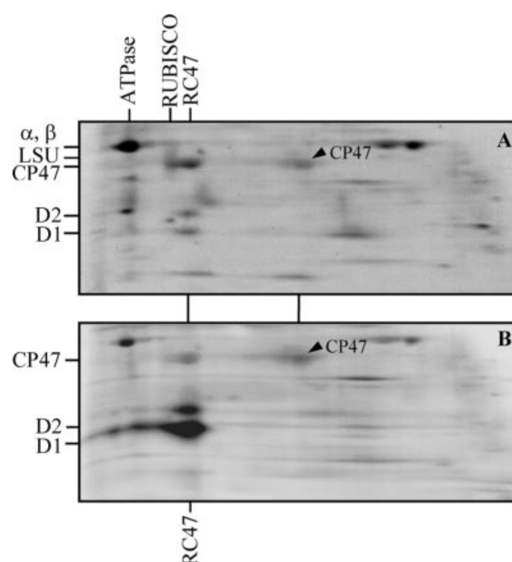

FIG. 2. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psb*C deletion strain lacking CP43 (Δ*psb*C).** Thylakoid membrane proteins from Δ*psb*C cells were radiolabeled, separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Coomassie stain, *A*; radiolabeling, *B*. Designation of proteins are as described in the legend to Fig. 1.

The RC47 complex itself was readily identified by the highly selective assembly of D1 into this complex. Although D2 and D1 were equally well stained with Coomassie, D2 radiolabel accumulation was low, whereas D1 radiolabel intensity was high and comparable with the RCC1 radiolabel accumulation in WT cells (Fig. 2*B*). Hence, a continued high rate of D1 synthesis was required to maintain the low level of RC47 complex in the mutant. Interestingly, no RC complexes and no unassembled D1 molecules were observed on the autoradiogram indicating that the high rate of D1 turnover could result in replacement of damaged D1 protein directly within RC47 (Fig. 2*B*). In addition, although the rate of D2 expression was low, accumulation of CP47, D1, and D2 and their assembly into RC47 was not blocked in general in the absence of CP43 expression. Data suggested that expression of *psb*C or accumulation of CP43 may be coupled to the expression of *psb*D or stability of the D2

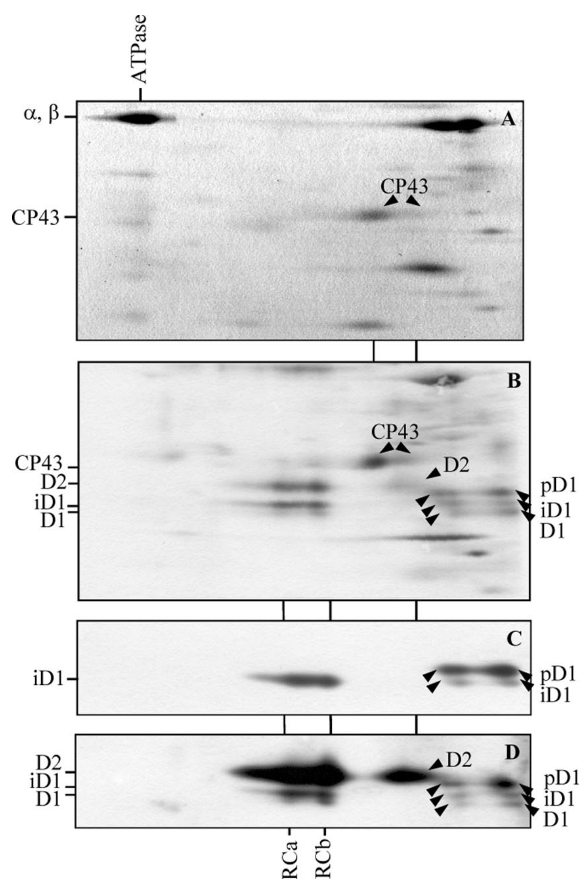

FIG. 3. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psb*B deletion strain lacking CP47 (Δ*psb*B).** Thylakoid membrane proteins from Δ*psb*B cells were radiolabeled, separated in the first dimension by a native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Coomassie stain, *A*; radiolabeling, *B*; Western blot using anti-pD1 extension, *C*; and Western blot using a mixture of anti-D1 and anti-D2 proteins, *D*.

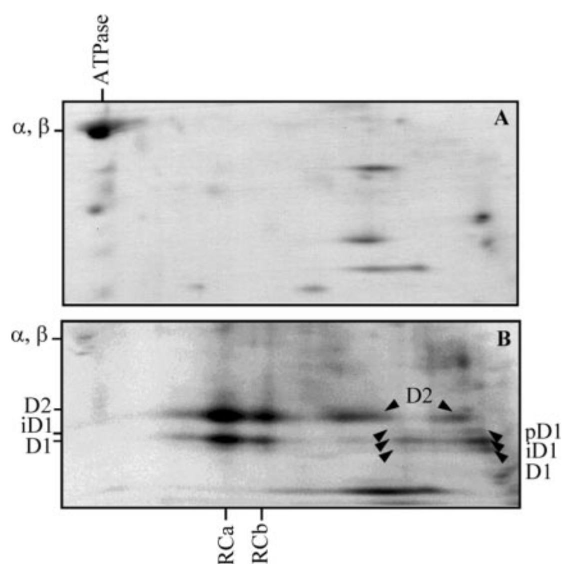

FIG. 4. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psb*B/*psb*C double deletion strain lacking CP47 and CP43 (Δ*psb*B/C).** Thylakoid membrane proteins from Δ*psb*B/C cells were radiolabeled, separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Coomassie stain, *A*; radiolabeling, *B*.

protein. We therefore tested next whether absence of CP47 in the presence of CP43 affects D2 expression and accumulation.

In psbB Deletion Strains, Two RC Complexes Accumulate—The two-dimensional BN/SDS gel Coomassie pattern of protein subunits from mutant thylakoids in the absence of CP47 revealed that two CP43 complexes, CP43a and CP43b, with decreasing molecular masses of 70–90 kDa were detectable. Furthermore, ATPase and Rubisco were still assembled indicating that assembly was not generally affected (Fig. 3*A*).

When *de novo* expression and assembly of PSII subunits was analyzed in the mutant by pulse radiolabeling, D2 and iD1 proteins were found to assemble in RCa and RCb demonstrating that in the absence of CP47 expression, the RC complexes represent the immediate precursor complex for RC47 assembly (Fig. 3*B*). As already indicated by analysis of the wild type, accumulation of radiolabeled matured D1 was drastically decreased in RCa and RCb and modified unprocessed iD1 was dominant here (Fig. 3*B*). The iD1 protein was also found in the region of unassembled proteins together with D2, pD1, and matured D1. Interestingly, all three D1 species as well as D2 were found in the form of a double band (Fig. 3, *C* and *D*). The pD1 and iD1 forms were detected by a C-terminal anti-extension antibody (Fig. 3*C*) and by an N-terminal antibody (Fig. 3*D*), indicating that pD1 and iD1 represent D1 precursor proteins with separate electrophoretic mobility. Whereas iD1 was found in both RC complexes and in the unassembled protein fraction, pD1 was present only in the free form (Fig. 3*C*). Gel blot analysis with a D1-specific antibody demonstrated the specific accumulation of iD1 in the RCa and RCb complexes. This supports our finding from wild type cells that the presence of the iD1 form indicates a slow rate of maturation of this D1 form within the RC complexes. Besides an identification of D2 in RC complexes RCa and RCb, gel blot analysis also demonstrated that in the *psbB* mutant, D2 accumulated in a yet unidentified complex at around 70 kDa characterized by an absence of D1 (Fig. 3*D*). To control these results, we constructed and analyzed a strain lacking both genes, *psb*B and *psb*C. Again, expression of D2 and D1 and their assembly in complexes RCa and RCb was comparable with the *psb*B mutant and D2 accumulated in the 70-kDa complex indicating that neither expression nor accumulation of CP47 nor CP43 was required for assembly of these complexes (Fig. 4, *A* and *B*). However, the Δ*psb*B/C deletion mutant was characterized by a higher RCa/RCb ratio and the absence of mature D1 in both RC complexes. These controls corroborated our finding that CP47 influences PSII assembly by binding, most likely, to the RCa complex. Hence, assembly of the RC complexes was investigated further.

In the psbA Deletion Strain, CP43, CP47, and D2 Are Expressed but Do Not Assemble—In the absence of *psb*A expression, the assembly machinery for ATPase and Rubisco remained fully functional, and both CP47 and CP43a complexes accumulated in the membrane; however, no higher molecular mass PSII complex could be found in the mutant thylakoids (Fig. 5*A*). More sensitive pulse-chase radiolabeling experiments revealed that *de novo* expressed CP43 accumulated as per usual in the two forms, CP43a and CP43b (Fig. 5*A*, *arrows CP43*). Again, as indicated from gel blot analysis (Figs. 3*D*, 4*B*, and 8*D*), D2 was found as a double band (*oblique arrows*) clearly identified by radiolabeling in the absence of D1 (Fig. 5*B*, *D2*, *arrow*). However, no further PSII intermediate complexes were detectable. This demonstrated that binding of D2 to iD1 is absolutely necessary for continued PSII assembly. Results were also remarkable with respect to the question of coupled expression of PSII subunits. Clearly, a lack of *psb*A expression did not down-regulate expression of *psb*B, *psb*C, or *psb*D or

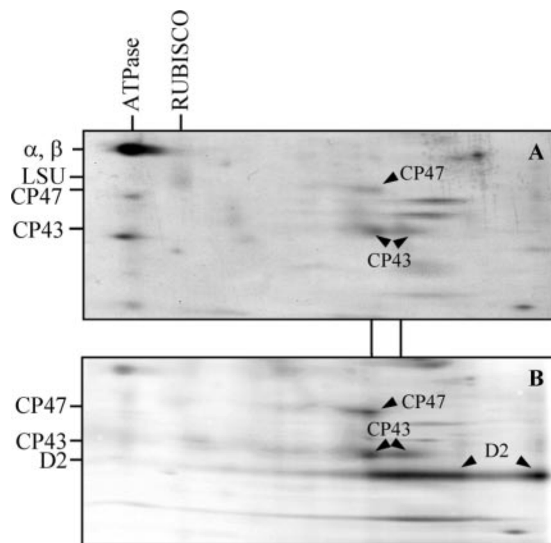

FIG. 5. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psb*A triple deletion strain lacking D1.** Thylakoid membrane proteins from Δ*psb*A cells were radiolabeled, separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in a 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Coomassie stain, *A*; radiolabeling, *B*.

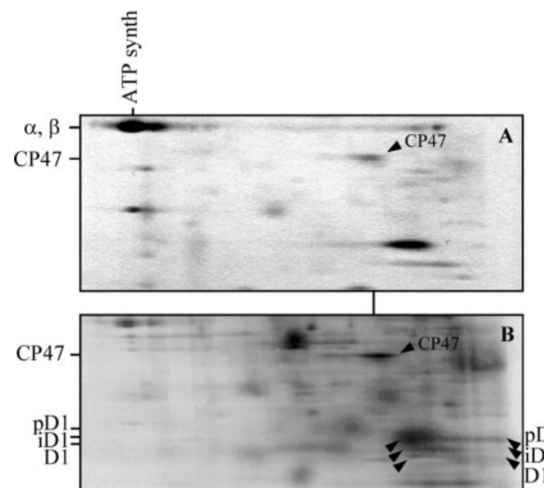

FIG. 6. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psbD*IC/*psbD*II double mutant lacking D2 and CP43.** Thylakoid membrane proteins from Δ*psbD*/C cells were radiolabeled, separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Coomassie stain, *A*; radiolabeling as described in the legend to Fig. 1*D*, *B*.

stability of the corresponding proteins in *Synechocystis* cells. Hence, the absence of D1 inhibited assembly, but did not block expression of the PSII subunits. We then tested the influence of D2 on expression and assembly of the assembly partners.

CP47 and D1 Are Expressed in a psbDIC/psbDII Deletion Strain—In the Δ*psb*D/C strain lacking expression of D2 and CP43, CP47 and ATP synthase were readily detected by Coomassie staining. Radiolabel incorporation demonstrated that expression of D1 and CP47 was not blocked although strongly decreased (Fig. 6, *A* and *B*). By pulse-chase radiolabeling, the D1 protein was identified in three separate double bands pD1, iD1, and D1 at about 70 kDa (Fig. 6*B*, *oblique arrows*) (Fig. 6*B*). Results showed that in the absence of D2, synthesis of CP47 and pD1, as well as processing of pD1 is possible. Comparison between protein labeling in the Δ*psb*D/C mutant and in the other studied strains showed that in general the synthesis of membrane proteins is reduced in this mutant.

Expression of the psbEFLJ Operon Is Selectively Required for psbD Expression and RC Precomplex Assembly—When *Synechocystis* cells without the *psb*EFLJ operon were analyzed by two-dimensional BN/SDS-PAGE, no assembly of RC complexes was found, although accumulation of ATPase was normal. Interestingly, we found by Coomassie staining and radiolabel analysis that CP43 and also CP47 accumulated as double bands in the 70–90-kDa region ((Fig. 7, *A* and *B*). Most remarkably, the expression pattern demonstrated that no synthesis of D2 could be detected, whereas expression of *psb*A remained high and double bands of all three D1 forms were identified in the region of unassembled proteins (Fig. 7*B*). In addition, we identified two weak radiolabeled complexes containing iD1 and unidentified components at around 100 kDa (Fig. 7*B*, *vertical arrows*). When we compared the D1 level in Δ*psb*EFLJ and wild type, almost no accumulation of D1 protein was detected in the mutant indicating that D1 is unstable in the mutant (Fig. 7*C*). Similar D1 levels were found by gel blot analysis in Δ*psb*D/C (not shown, see Fig. 8*E*). In contrast, the level of D2 in Δ*psb*A reached about 15–20% of the wild type level. This suggests that D2 is more stable in the absence of D1 than D1 in the absence of D2. We concluded that expression of the *psb*EFLJ operon is a prerequisite for synthesis of the D2 protein and consequently for assembly of RC complexes. To further investigate the function of the cytochrome b_{559} subunit we then analyzed the presence of cytochrome b_{559} in protein complexes.

Cytochrome b_{559} Assembles with a D2/D1 Complex in the Absence of CP47—Thylakoid membrane proteins from various mutants were transferred onto polyvinylidene difluoride membrane and probed with antibodies specific for the α subunit of cytochrome b_{559}, because the protein was not detected by Coomassie staining and was also not readily radiolabeled *in vivo*. Antibodies directed against proteins D1 and D2 were used to estimate protein accumulation and to detect assembly intermediates of these proteins with cytochrome b_{559}. In the wild type, the cytochrome subunit was readily found in RCC1 as well as in RC47 (Fig. 8*A*). In addition, the protein was also present in a broad molecular mass range between 50 and 70 kDa that was identified in all analyzed strains with exception of the *psb*EFLJ deletion mutant. The α-cytochrome subunit therefore migrates at a higher molecular mass than predicted from its sequence, even when expected to form a heterodimer with the β-subunit. In Δ*psb*C, the subunit was found in RC47 (Fig. 8*B*). In Δ*psb*B, immunodetection showed its presence in both RCa and RCb complexes (Fig. 8*C*). In the *psb*A deletion mutant, the position of cytochrome b_{559} on the blot was comparable with that of the D2 protein band that was detected as an asymmetrical double band in a molecular mass range between 50 and 100 kDa (Fig. 8*D*, *oblique arrows*). The cytochrome band was also asymmetrical but with a different shape as compared with D2. Nevertheless, unlike the other strains there was a more distinct part of the band with higher molecular size that could represent the D2-cytochrome b_{559} RC precomplex similar to that found in higher plants (40). The cytochrome band in the 50–70-kDa region was also found in the Δ*psb*D/C strain showing that synthesis of cytochrome b_{559} and accumulation of this complex was independent from *psb*D expression (Fig. 8*E*, Δ*psbD/C*). In contrast to Δ*psb*A, in Δ*psb*D/C no partially separated cytochrome subcomplex was detected at higher molecular mass. In the *psb*EFLJ deletion mutant, almost no accumulation of D1 and D2 was detectable, which confirmed the requirement of cytochrome b_{559} for biogenesis of PSII (Fig. 8*F*).

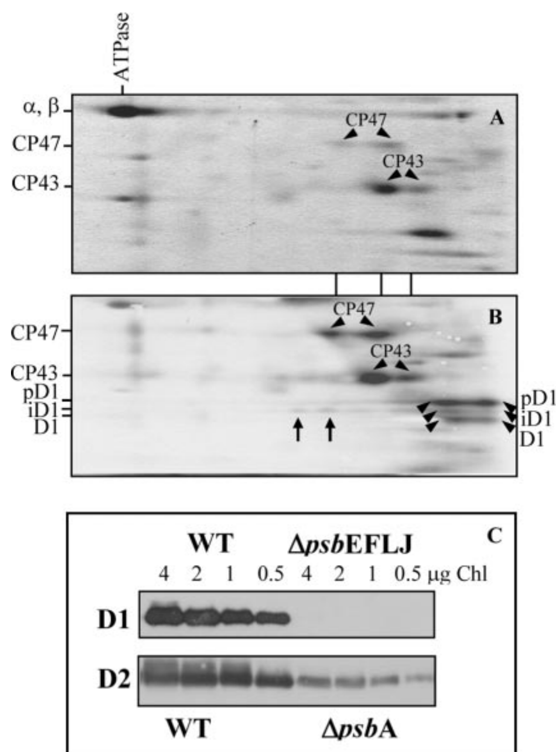

FIG. 7. **Two-dimensional BN/SDS-PAGE analysis of a *Synechocystis psb*EFLJ deletion mutant lacking cytochrome b_{559} subunits and small proteins PsbL and PsbJ (*A* and *B*) and estimation of D2 and D1 content in thylakoids of Δ*psb*A and Δ*psb*EFLJ strains (*C*).** *A* and *B*, thylakoid membrane proteins from Δ*psb*EFLJ cells were radiolabeled, separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in a 12–20% polyacrylamide gel and stained as described under "Materials and Methods." Designation of proteins are as described in the legend to Fig. 1. Putative complexes of iD1 with unknown components are marked by *vertical arrows*. Coomassie stain, *A*; radiolabeling, *B*. *C*, samples of thylakoids from WT, Δ*psb*A, and Δ*psb*D/C strains containing 4, 2, 1, and 0.5 μg of chlorophyll were analyzed for proteins by PAGE, transferred onto nitrocellulose membrane, and probed by antibodies raised against D2 and D1 proteins as described under "Materials and Methods."

DISCUSSION

De novo assembly of PSII subunits was analyzed in wild type and mutant cyanobacterial cells lacking expression of structural PSII protein subunits. Because the protein subunit composition present in the functional PSII complex is known from crystallization studies, we aligned PSII subcomplexes isolated from the mutant cells according to molecular mass. The highest molecular mass complex that assembled despite the absence of a structural subunit was interpreted as an assembly intermediate awaiting the missing structural subunit. The assembly intermediates from all mutants then present an assembly map of PSII. Our data indicate a stepwise assembly of the structural PSII protein subunits.

Interdependence of the PSII Subunit Expression in Synechocystis—Our study of the *psb*EFLJ deletion strain revealed that expression and accumulation of cytochrome b_{559} constitutes an initial step for synthesis of the D2 protein, whereas synthesis of the D1, CP47, and CP43 proteins is independent of cytochrome b_{559}. Regulation of D2 synthesis by the presence of cytochrome b_{559} may correspond to a type of regulation previously described for cytochrome b_6f assembly in the green alga *Chlamydomonas*. The absence of subunit IV of the cytochrome b_6f complex was shown to down-regulate the synthesis of the cytochrome *f* protein (51). This concept of epistatic synthesis may prevent wasting energy for the synthesis of proteins that cannot be assembled. If a similar mechanism exists in *Synechocystis*, expression of the concept of epistatic synthesis protein D2 would be down-regulated during absence of the regulatory assembly partner cytochrome b_{559}.

In this respect, absence of the *psb*DIC and *psb*DII genes caused a general decrease in synthesis of the majority of membrane proteins (Fig. 6*B*), whereas absence of *psb*A expression did not lead to apparent down-regulation of PSII subunits CP43 and CP47. This suggests that the synthesis of the D1 protein is independent on the other PSII components and its accumulation is mostly dictated by the rate of its degradation as suggested by results from the study of Δ*psb*D/C *and* Δ*psb*EFLJ.

Role of Cytochrome b_{559} in the de Novo Assembly of PSII—In barley etioplasts, the formation of an RC assembly precomplex containing D2 and cytochrome b_{559} was shown to precede PSII assembly (40). In *Synechocystis*, the synthesis and assembly state of the D1, D2, and cytochrome b_{559} proteins in the *psb*B, *psb*DIC/*psb*DII, and *psb*EFLJ deletion strains showed that the presence of cytochrome b_{559} is a prerequisite for D2 synthesis and accumulation, making it likely that a D2-cytochrome b_{559} precomplex may represent an initial assembly unit from which the PSII assembly starts in *Synechocystis* (Fig. 9). The existence of such a precomplex is suggested from gel blot analysis of the Δ*psb*B and Δ*psb*A strains (Figs. 3*D* and 8*D*). In contrast to others, we show that in *Synechocystis* synthesis of the D1 protein is an independent process that neither requires the presence of D2, nor of cytochrome b_{559} in the membrane (39); however, we agree with work in which D1 incorporation into PSII has been postulated to be dependent on the presence of the D2 protein (41). According to our data set, assembly of D2 with cytochrome b_{559} is a prerequisite for assembly of pD1 into a RC complex that stabilizes pD1 against rapid degradation. Whether insertion of the complete pD1 protein into the membrane phase precedes its assembly with the D2-cytochrome b_{559} precomplex or whether the protein is cotranslationally associated with the D2-cytochrome b_{559} precomplex during its synthesis remains open (52) (Fig. 9).

Gel blot analysis showed that in all studied strains (with exception of Δ*psb*EFLJ) the α-subunit of cytochrome b_{559} is present in a relatively large complex outside of RCC and RC complexes (Fig. 8) and this complex also contains the β subunit (data not shown). In WT and Δ*psb*C, it is apparent that no other large PSII subunits are present in the unassembled state excluding the possibility that this cytochrome band is a result of PSII disassembly during solubilization or electrophoresis. Therefore, a significant fraction of cytochrome b_{559} resides in the thylakoid membrane free of other PSII subunits. The importance of this finding is not clear but such a fraction could represent a pool for immediate initiation of the *de novo* assembly of PSII upon demand. Another possibility is that this cytochrome b_{559} species may be a general heme reservoir that can be promptly utilized by cyanobacterial cells whenever it is needed.

Reutilization of Subunits during PSII Assembly—In the WT strain, incorporation of radiolabel is highest in the D1 protein, because the rate of D1 turnover related to the PSII repair mechanism is high (12, 13). Labeling of D2 and CP43 is weaker but significant suggesting turnover of these proteins. On the other hand, CP47 labeling is very low indicating that this protein is reused in several assembly/disassembly cycles before being turned over. Interestingly, the degree of radiolabel found in accumulated PSII proteins in *Synechocystis* cells *in vivo* correlates well with that obtained in isolated spinach chloroplasts, indicating that it may directly relate to levels of protein damage and inversely reflect the frequency of reutilization of

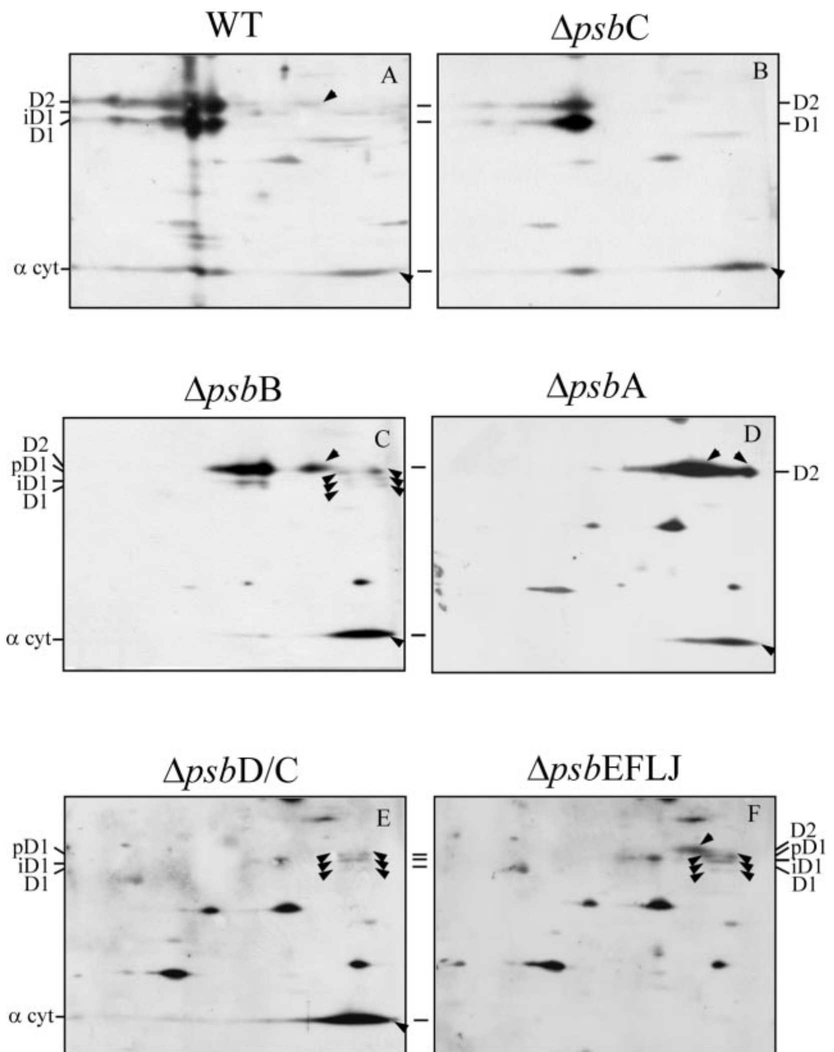

FIG. 8. **Gel blot analysis of *Synechocystis* strains after separation of thylakoid membrane proteins by two-dimensional BN/SDS-PAGE.** Thylakoids were isolated from the following *Synechocystis* strains: WT (*A*); *psb*C, lacking CP43 (*B*); Δ*psb*B, lacking CP47 (*C*); Δ*psb*A, lacking D1 (*D*); Δ*psb*D/C, lacking D2 and CP43 (*E*); and Δ*psb*EFLJ, lacking cytochrome b_{559}, L and J subunits (*F*). Thylakoid proteins were separated in the first dimension by native BN-PAGE and in the second dimension by denaturing SDS-PAGE in the 12–20% polyacrylamide gel, transferred onto nitrocellulose membrane, and probed with antibodies raised against D1, D2, and the α subunit of cytochrome b_{559} as described under "Materials and Methods." *α cyt*, α subunit of cytochrome b_{559}, other designations are identical as described in the legend to Fig. 1.

PSII proteins in the organisms (39, 41). Under conditions of inhibited repair, this was shown in the thermophilic cyanobacterium *Synechococcus*, where the probability of damage is decreasing in the order D1 ≥ D2 > CP43 > CP47 (53).

Selective Replacement of D1 Predominantly Occurs in RC47—In the *psb*C deletion strain, the RC47 complex is the dominant assembly intermediate in which D1 accumulates. The complex has been found in similar mutants (54, 55) and a significantly elevated *psb*A transcript level had been noted (24). Our finding that the highest label in D1 is observed in the RC47 complex while only a minimal amount of the D1 protein was found in RC complexes as well as in the free fraction is remarkable. Taking into account a much lower steady state level of the D1 protein in the mutant that should not exceed 20% of the WT level (54) such intensive labeling indicates an extraordinary high reassembly of this protein into the RC47 complex (Fig. 9). We therefore conclude that the absence of any radiolabel accumulation in RC complexes and free D1 may indicate a direct, selective cotranslational or an indirect fast post-translational replacement of damaged D1 within the RC47 complex. Because we were not able to detect any residual pD1 in this complex, processing of *de novo* expressed pD1 appeared to be rapid as previously seen (53, 56). To achieve a preferential targeting of the D1 protein into the RC47 complex, we expect that factors crucial for targeting and replacement are associated with the RC47 complex or the D1 protein. These may be either PSII components that are exposed in the absence of CP43, or external proteins with high affinity to RC47 or to the *de novo* expressed and membrane-integrated D1. For D1, candidates to regulate the *de novo* assembly are Alb3, which is required in higher plant chloroplasts for efficient assembly of D1 directly after release to the membrane phase (57), hcf136, which has been indicated to associate with D2 and cytochrome b_{559} during RC complex formation (58), and the FtsH protease associated with the prohibitin complex, which has been proposed to be responsible for the selective replacement of the D1 protein (59). Also, a role of the Sec translocon and the involvement of chaperones like HSP70 cannot be excluded (52).

Formation of Photosystem Complexes with Non-PSII Proteins in the Absence of the PSII Binding Partner—In the absence of CP47, synthesis of D1, D2, and CP43 but only accumulation of CP43 had been shown in *Synechocystis* cells (23). Our finding that in the absence of CP47 assembly is halted on the level of the RC complex demonstrates that CP43 is not capable of forming a complex with the RC complex (Figs. 3 and 4). Therefore, we conclude that the D1 side of the RC complex is blocked for CP43 assembly, if CP47 is not bound to the D2 side of the RC complex. This block could be caused by improper conformation of the D1 side in the absence of CP47, or by a protein factor bound to the D1 side. Our finding that the RCa band extends toward the higher molecular mass region where the RC47 complex is found in WT cells indicates such a regulatory protein binding (Fig. 3, *B–D*). Binding of other non-PSII proteins to particular PSII subunits is also suggested by findings where protein complexes containing apparently just one labeled PSII protein are identified at significantly higher molecular mass

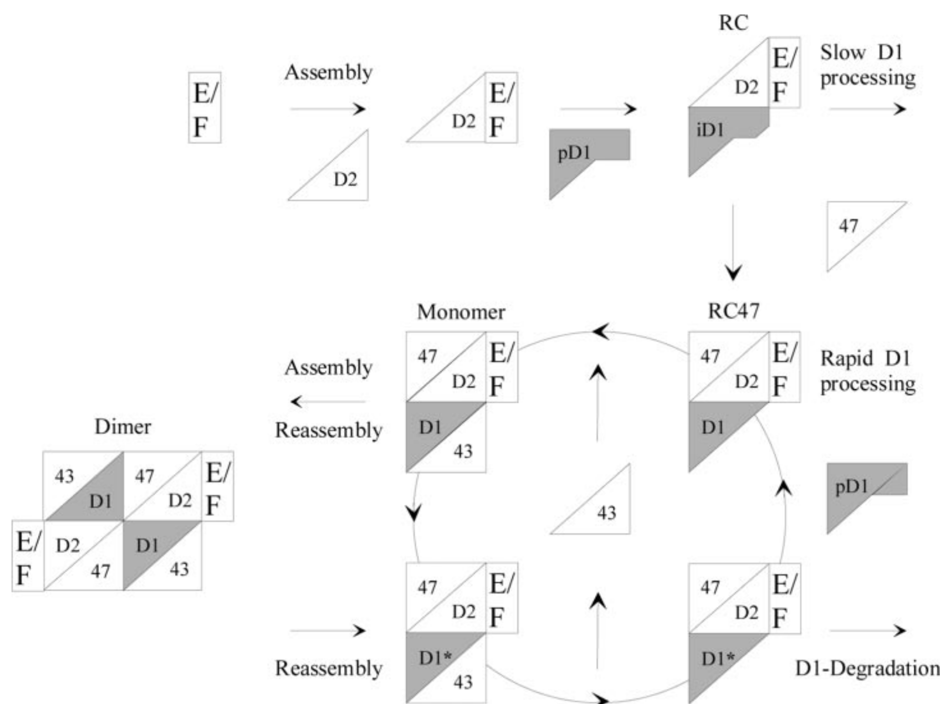

FIG. 9. **Model for PSII assembly in thylakoid membranes of *Synechocystis*.** Assembly and reassembly of PSII is displayed as a series of assembly steps (*arrows*) leading to consecutive association of protein subunits (genes) E/F (*psb*E/F) with D2 (*psb*D), pD1 (*psb*A), CP47 (*psb*B), and CP43 (*psb*C) (proteins displayed as *rectangles* and *triangles*) during assembly of PSII reaction center core monomers (*Monomer*) and dimerization of monomers (*Dimer*). Inactivation of D1 in monomers and dimers (D1*) initiates a reassembly cycle leading to detachment of CP43 from monomeric PSII (*Monomer*) and formation of the RC47 complex (*RC47*), replacement of D1* by pD1, processing of pD1 within this complex (*RC47*), reassembly of CP43 onto the RC47 complex (*Monomer*), and consecutive dimerization of the reaction center core monomers (*Dimer*).

than the majority of the protein. Although, we cannot completely exclude that these complexes represent aggregates because of the absence of their proper PSII binding factors, results for CP43 in the Δ*psb*B strain (Fig. 3) and for the iD1 protein in the Δ*psb*EFLJ strain indicate that accumulation of the distinct bands represent specific complexes (Fig. 7, *vertical arrows*). Unfortunately, these complexes were detected only by radiolabeling and their negligible amount did not allow us to identify their protein composition by mass spectrometry.

Is Binding of Small Proteins or Pigments to Chlorophyll Proteins Resolved by two-dimensional BN-PAGE?—Native electrophoresis clearly showed that in unassembled or partially assembled states all four radiolabeled PSII chlorophyll proteins, D1, D2, CP47, and CP43, can exist in two forms clearly differing by their mobility in the native gel. The two forms either represent a single protein at mobility and a specific precomplex with an additional small protein subunit that is responsible for the molecular mass increase, or both forms may still represent single apoproteins, differing in molecular mass because of binding of pigments, lipids, or other prosthetic groups. After preincubation of WT cells in the presence of gabaculine to block chlorophyll biosynthesis, the accumulation of radiolabel increased in RCb but not RCa, more CP43b than CP43a accumulated, and D2 was detected merely in lower M_r bands indicating an assignment of the lower M_r bands to complexes not associated with chlorophylls (data not shown). Also, after 16 h preincubation of WT cells in the presence of 5 μM fluridon, an inhibitor of carotenoid synthesis, the ratio between the higher and lower molecular weight forms of CP43 (CP43a *versus*CP43b) was markedly decreased (data not shown). These findings were corroborated in the Δ*psb*B/C strain, where less RCb than RCa accumulated suggesting that in the absence of CP47 and CP43, a higher availability of Chl for binding to RC complexes may have resulted in accumulation of the higher molecular mass RC form. Because assembly of RC complexes is possible in the absence and presence of Chl, we conclude that in *Synechocystis*, loading of the RC complex with Chl may occur after assembly of the protein subunits.

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Localization of the PsbH subunit in photosystem II from the *Synechocystis* 6803 using the His-tagged Ni–NTA Nanogold labeling

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Abstract

The PsbH protein belongs to a group of small protein subunits of photosystem II (PSII) complex. This protein is predicted to have a single transmembrane helix and it is important for the assembly of the PSII complex as well as for the proper function at the acceptor side of PSII. To identify the location of the PsbH subunit, the PSII complex with His-tagged PsbH protein was isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 and labeled by Ni^{2+}–nitrilo triacetic acid Nanogold. Electron microscopy followed by single particle image analysis identified the location of the labeled His-tagged PsbH protein at the periphery of the dimeric PSII complex. These results indicate that the N terminus of the PsbH protein is located at the stromal surface of the PSII complex and close to the CP47 protein.
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Keywords: Electron microscopy; Photosystem II; PsbH protein; Immunogold labeling

1. Introduction

Oxygenic photosynthesis is a process in which plants, algae and cyanobacteria use light energy to drive the synthesis of organic compounds and produce all molecular oxygen, necessary for aerobic life on Earth. The light-harvesting and energy-transducing functions of oxygenic photosynthesis are localized in specialized photosynthetic membranes, thylakoids, and carried out by several types of protein complexes embedded in the membrane. Central to this process is photosystem II (PSII) complex that catalyzes the light-induced production of oxygen, thereby transferring electrons from water to plastoquinone. The PSII complex is dimer and contains more than 25 subunits per monomer (Hankamer et al., 2001a). All redox cofactors are bound to the central part of the complex formed by the reaction center D1 and D2 proteins. The reaction center is surrounded by the so-called inner antenna proteins CP43 and CP47, and several low molecular mass subunits each predicted to have a single transmembrane helix.

PsbH protein belongs to a group of small protein subunits of the PSII complex. The PsbH protein was originally detected as a 10 kDa phosphoprotein (Bennet, 1977) and subsequently sequenced in number of prokaryotic and eukaryotic organisms (recently reviewed by Komenda et al., 2003). The PsbH protein is predicted to have single transmembrane helix with its 72 amino acid residues in higher plants (Shinozaki et al., 1986) and 87 amino acid residues in the green alga *Chlamydomonas reinhardtii* (Dedner et al., 1988).

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In higher plants and green algae the PsbH protein undergoes reversible phosphorylation at two threonine residues close to the N terminus (Michel and Bennett, 1987; Vener et al., 2001). The cyanobacterial PsbH protein is truncated at its N terminus and misses these phosphorylation sites (Mayes and Barber, 1991). The function of PsbH protein in PSII complex has been associated, through analysis of a *Synechocystis* mutant lacking the coding gene, with control of the electron flow from Q_A to Q_B (Mayes et al., 1993), protection from photoinhibition (Komenda and Barber, 1995), and bicarbonate binding on its acceptor site (Komenda et al., 2002). However, disruption of the PsbH subunit in *C. reinhardtii* led to the disappearance of PSII from thylakoid membrane (O'Connor et al., 1998; Summer et al., 1997).

Recently, three-dimensional (3-D) structures of the PSII core complex have been solved by X-ray (Ferreira et al., 2004; Kamiya and Shen, 2003; Zouni et al., 2001) and electron crystallography (Hankamer et al., 2001b). The models show that the overall organization of 22 transmembrane helices of the major PSII subunits (CP47, CP43, D1, and D2) is preserved between higher plant and cyanobacteria. In addition, 12 (spinach) and 14 (cyanobacteria) low molecular mass subunits, each predicted to have single membrane-spanning α-helix, were identified in each monomer of the dimeric PSII complex. Of them only PsbE and PsbF, as components of cytochrome (cyt) b_{559}, were identified by locating the haem group near to D2 protein of the D1/D2 reaction center complex. So far, the assignment of the smaller subunits in PSII is not certain and have been based on chemical cross-linking studies (Büchel et al., 2001; Hankamer et al., 2001a), immunogold labeling experiments (Bumba and Vácha, 2003) or comparative studies of wild type with mutants depleted of the smaller PSII proteins (Komenda et al., 2002; Shi et al., 2000; Swiatek et al., 2001). Unfortunately, to date, all attempts to work out the location of the small subunits rationally have generated contradictory information (Shi and Schröder, 2004). Recently, Büchel et al. (2001) identified the location of the PsbH subunit in PSII core complex from *C. reinhardtii* using a His-tagged PsbH mutant and gold labeling. Here, we describe the Ni^{2+}–nitrilo triacetic acid (Ni–NTA) Nanogold labeling of the PsbH subunit in the PSII core complex isolated from the His-tagged PsbH mutant of the cyanobacterium *Synechocystis* 6803. The gold label consists of a gold cluster coupled to Ni–NTA Nanogold that has a high binding affinity to multiple histidine residues of the His-tagged protein (Hainfeld et al., 1999). In combination of single particle analysis of Ni–NTA Nanogold labeled PSII particles and molecular modeling of known X-ray coordinates of the PSII structure we were able to suggest the location of the PsbH subunit within the PSII complex.

2. Materials and methods

2.1. Mutant construction

The *Synechocystis* 6803 PsbH-His strain with PsbH protein tagged with the His_6 epitope on its N terminus expressed under psbA2 promoter has been constructed. *PsbH* gene was amplified by PCR using the mix of *Taq* and *Pfu* DNA polymerases and gene specific primers with artificially generated restriction sites for *Nde*I and *Bam*HI and containing six histidine codons (CAT) in the forward primer. After restriction, the PCR fragment was cloned into *Nde*I and *Bam*HI sites of the pSBA plasmid containing the upstream and downstream regions of the *Synechocystis* 6803 *psb*AII gene (Lagarde et al., 2000). Ligation mix was amplified by PCR using the pSBA primers amplifying the whole *psbA*/*psbH-His* region. Amplification by PCR was chosen because transformation of *Escherichia coli* with the ligation mix repeatedly yielded no colonies. The PCR product containing *psb*H-His gene was transformed into *Synechocystis* 6803 psbAII-KS strain where the psbA2 gene was replaced by kanamycin-resistance/*sacB* cartridge (Lagarde et al., 2000). The *sacB* gene is coding for levan sucrase leading to sucrose sensitivity of this strain. After transformation, *Synechocystis* cells were grown on BG-11 plates for four days. Transformants were than transferred to plates with 5% sucrose and sucrose resistant colonies were checked for kanamycin sensitivity. Resulting strain expressing both wild type and His tagged forms of PsbH protein has been transformed with chromosomal DNA from $PsbH^-$ strain (Mayes et al., 1993). Deletion of wild type copy of *psbH* gene in PsbH-His strain was confirmed by PCR.

2.2. Isolation of thylakoid membranes

Cells were harvested in the exponential growth phase, resuspended in thylakoid buffer (50 mM MES/NaOH, pH 6.5) and broken in a *MiniBeadBeater* (BioSpec Products, USA) by three breaking cycles (30 s shaking followed by a 3 min cooling on ice). After centrifugation at 2000*g* for 1 min to remove cell debris, the supernatant was centrifuged in at 30,000*g* for 10 min at 4 °C. Thylakoid membranes were resuspended in thylakoid buffer.

2.3. Isolation of PSII complex

Thylakoid membranes were solubilized with 1% dodecyl-maltoside (DM) in thylakoid buffer with 100 mM NaCl at chlorophyll concentration of 1 mg ml^{-1} for 15 min. The unsolubilized material was removed by centrifugation for 30 min at 60,000 *g* and the supernatant was used for affinity chromatography on Fractogel EMD Chelate (Merck) charged with Ni^{2+}. The column was sequentially washed with thylakoid buffer–NaCl

with 5 and 25 mM imidazole. PSII was eluted by 50 mM imidazole. The PSII was further purified by gel filtration chromatography on Superdex 200HR 10/30 column (Amersham Biosciences) connected to a HPLC pump (LCP 3001, Ecom, Czech Republic) and photodiode array detector Waters 996 (Waters, USA). The column was equilibrated with 20 mM MES/NaOH (pH 6.5), 10 mM NaCl and 0.03% DM at flow rate of 0.5 ml/min (Bumba et al., 2004).

2.4. Polyacrylamide gel electrophoresis and Western blotting

Protein composition was determined by SDS–PAGE using a 12–20% linear gradient of polyacrylamide gel carried out using Tris/glycine gel containing 7 M urea. Gels were stained with Coomassie brilliant blue stain. After electrophoresis, proteins were transferred to *PVDF* membrane (*Hybond-P*, *Amersham Biosciences*) using wet blotting system. Blotted membranes were subjected to immunoblot analysis using the PsbH antibody (1:6000 dilution) or *polyHistine* antibody (*Sigma H 1029*, 1:3000 dilution). Primary antibody was detected with peroxidase-conjugated secondary antibody. PsbH antibody has been prepared in rabbit using PsbH-GST fusion protein expressed in *E. coli* as an antigen (Halbhuber et al., 2003).

2.5. Pigment analysis

Room temperature absorption spectra were recorded with a UV300 spectrophotometer (Spectronic Unicam, UK). Fluorescent emission spectra were measured at liquid nitrogen temperature using a Fluorolog spectrofluorometer (Jobin Yvon, USA) with an excitation wavelength of 430 nm.

2.6. Oxygen evolution

Oxygen evolution was measured using a Clark-type oxygen electrode (Hansatech). Samples at a chlorophyll concentration of 10 μg(Chl) ml^{-1} were suspended in a medium containing 20 mM MES (pH 6.5), 0.3 M sucrose, 20 mM $CaCl_2$, 10 mM $NaHCO_3$, 10 mM NaCl, supplemented with electron acceptors, 2,5-dichloro-*p*-benzoquinone at a concentration of 500 μM and ferrycyanide at a concentration of 2.5 mM and illuminated with saturating white light.

2.7. Electron microscopy and image analysis

Freshly prepared PSII complexes were placed on glow-discharged carbon coated copper grids and excessive liquid was removed using filter paper. The grid was then placed upside-down on a droplet of a Ni^{2+}-nitrilotriacetic acid Nanogold (Ni–NTA Nanogold) solution (Nanoprobes, USA). After 15 min incubation at room temperature the grid was removed from the droplet, rinsed with water and negatively stained with 0.75% uranyl acetate. Electron microscopy was performed with Philips TEM 420 electron microscope using 80 kV at 60,000× magnification. Micrographs free from astigmatism and drift were scanned with a pixel size corresponding to 4.5 Å at the specimen level. Image analyses were carried out using SPIDER software (Frank et al., 1996). From 85 micrographs of the PSII cores, about 3500 top-view projections of unlabeled particles and 260 top-view projections of labeled particles were selected for analysis. Both separate data sets were rotationally and translationally aligned, and treated with multivariate statistical analysis in combination with classification (Harauz et al., 1988; van Heel and Frank, 1981). Classes from each of the subsets were used for refinement of alignments and subsequent classifications. For the final sum, the best of the class member were summed, using a cross-correlation coefficient of the alignment procedure as a quality parameter. The resolution of the images was calculated by using the Fourier ring correlation method (van Heel, 1987). For molecular modeling, the coordinates were taken from Protein Data Bank (www.rcsb.org/pdb) under the code 1S5L for PSII structure at 3.5 Å resolution (Ferreira et al., 2004). The overlay cartoon was generated by freeware program Accelrys ViewerLite 4.2.

3. Results

3.1. Biochemical characterization of the PsbH-His PSII complex

Functional integration of the PsbH protein tagged with the His_6 epitope on its N terminus into PSII in PsbH-His strain of *Synechocystis* 6803 has been confirmed by high light treatment. Unlike $PsbH^-$ strain, PsbH-His strain was able to grow at light intensity of 200 μmol (photons) $m^{-2} s^{-1}$ with growth rate comparable to that of wild type (not shown).

His-tagged PSII complex was purified by a single-step Ni^{2+} affinity column chromatography. The PSII fraction was eluted with a concentration of 50 mM imidazole. The polypeptide composition of the purified PsbH-His PSII complex was analyzed by SDS–PAGE (Fig. 1). The PSII complex consisted of at least CP47, CP43, D2, D1, cytochrome b_{559} as confirmed by Western blot (not shown). The presence of PsbH protein in the PSII preparation was probed by immunodetection with anti psbH and anti His antibodies. As shown in Fig. 1, there was a clear shift (about 1 kDa) in the mobility of the PsbH-His protein in comparison to the PsbH caused by the six additional histidines.

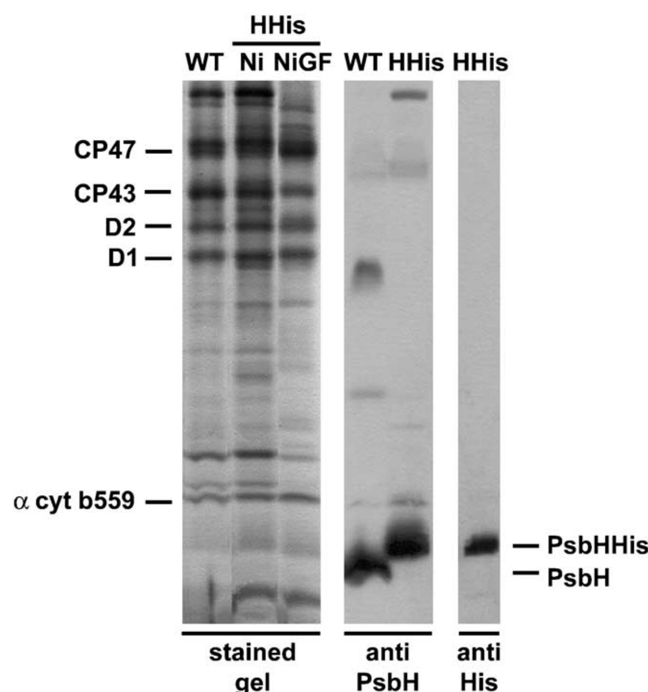

Fig. 1. Protein composition of PSII complex isolated from *Synechocystis* 6803. PSII complex purified from wild type (WT) and the mutant psbH-His strain (HHis) by Ni^{2+} affinity (Ni) followed by gel filtration chromatography (NiGF) was analyzed by SDS–PAGE. Proteins were either stained with Coomassie Blue (stained gel) or blotted onto PVDF membrane and immunodecorated using either antibody against PsbH-GST conjugate (anti-PsbH) or antibody against His_6 epitope (anti-His).

Room temperature absorption spectrum of the PSII fraction is shown in Fig. 2A. The PSII fraction exhibited absorption maxima at 438 nm and 674 nm and lacked the significant absorbance around 550 nm indicating that the sample is free of phycobiliproteins. Seventy-seven Kelvin fluorescence emission spectrum of PSII fraction showed a single emission peak with maximum at 692 nm characteristic for PSII complex (Fig. 2B). The PSII complexes were active in oxygen evolution and yielded $386 \pm 45\,\mu mol(O_2)\,mg(Chl)^{-1}\,h^{-1}$.

The PSII fraction was further purified by gel filtration chromatography (Fig. 2C). Gel filtration analysis of PSII fraction shows a major peak eluting at 18.2 min that corresponds to the dimeric PSII complex with a molecular mass of about 500 kDa. Small peaks eluting at 22 min and 26 min may be interpreted as monomeric PSII complex and free PSII proteins, respectively. For electron microscopy, the dimeric PSII complexes were collected from the maximum of the main peak of the gel filtration.

3.2. Electron microscopy and gold labeling of the PsbH-His PSII complex

To identify the location of the His-tagged PsbH subunit within PSII, the dimeric PSII complexes were immobilized on glow-discharged carbon-coated electron microscopy grid and labeled with Ni–NTA Nanogold (Büchel et al., 2001). The advantage of this labeling lies in a more accurate localization of the targeted site since no additional protein densities are present as compared with conventional immunogold labeling procedures. This approach gave specific labeling of multiple His sites on the protein complexes (Fig. 3). The effectiveness of the procedure was confirmed by carrying out the labeling in a buffer containing 30 mM imidazole which, due to competition with the His-tag for the Ni–NTA sites, abolished any labeling of proteins (Büchel et al., 2001).

Labeled PSII complexes were then negatively stained and visualized in electron microscope. A typical EM images in Fig. 3 clearly show that the preparation contains dispersed particles with uniform size and shape and almost free of contaminants. The image shows that the preparation contained dimeric PSII particles, mostly in their top-view projections (i.e. perpendicular to the original membrane plane). Fig. 3 also shows that only a few PSII dimers exhibited gold label. Although different labeling conditions, such as incubation time, label concentration, pH and temperature were employed to improve gold labeling, no significant changes in the extent of labeling were observed (not shown).

3.3. Image analysis

To reveal the exact location of the gold label within PSII dimers, both labeled and unlabeled particles were extracted from the micrographs, and separately aligned, treated with multivariate statistical analysis and classified. The most representative class averages of both labeled and unlabeled particles are depicted in Fig. 4. Although no symmetry has been imposed during the image analysis clearly two-fold rotational symmetry around the center of the complex is visible. The class averages were similar in size and shape, and they closely resembled PSII core complexes without the His-tagged PsbH protein (Boekema et al., 1995; Bumba et al., 2004; Kuhl et al., 1999; Nield et al., 2000). All the projections had the same type of handedness and no mirror images were detected, thus indicating preferred orientation of the PSII dimers with their stromal side to the carbon support film. Therefore, the relatively low number of labeled particles probably reflects the preferential binding of the PSII particles. Since a His-tag of the PsbH protein is located on the stromal side of the complex, the labeling site is inaccessible when the particles are orientated with their stromal sides to the carbon support film. A final sum of labeled PSII core complexes is presented in Fig. 4E. The averaged top-view projection indicates a particle with single label located at the periphery of the complex. The resolution of the final projections was calculated by means of the Fourier ring correlation method (van Heel, 1987) and found to be 26 Å for class average

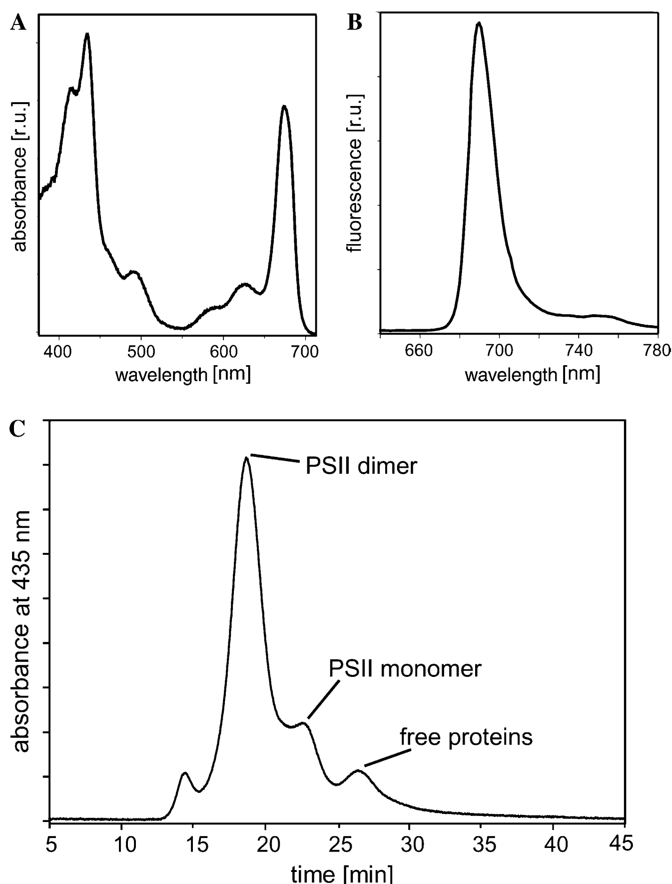

Fig. 2. Absorbance and fluorescence properties of PSII complex with His-tagged PsbH protein isolated from *Synechocystis* 6803: (A) room temperature absorbance spectrum; (B) fluorescence emission spectrum at 77 K excited at 430 nm; (C) gel-filtration chromatography elution profile of PSII fraction obtained from Ni^{2+} affinity column chromatography. The chromatogram was detected at 435 nm for chlorophyll containing proteins.

of gold labeled PSII dimers and 21 Å for unlabeled class averages.

4. Discussion

Here we present the study to identify the location of the PsbH protein within the PSII core complex. The combination of Ni–NTA Nanogold labeling of His-tagged protein and single particle analysis provides an excellent tool to localize protein subunits within a multi-subunit protein complex (Büchel et al., 2001). The advantage of this labeling lies in a more accurate localization of the targeted site since no additional protein densities are present as compared with conventional immunogold labeling procedures. Although low number of labeled particles was collected from the micrographs this approach gave specific labeling of His-tag sites, enabling us to locate the PsbH in the PSII core complex (Fig. 3).

Biochemical and spectroscopic characterization of isolated PSII complex with His-tagged protein revealed that incorporation of recombinant PsbH protein into PSII has no evident effect on its structure and function. The PSII dimers were active in oxygen evolution indicating the integrity of the extrinsic polypeptides located at the lumenal side of the complex. In addition, a comparison of the top-view projection map of the dimeric PSII complex isolated from the wild type with that of the PsbH-His PSII complex suggested identical size and shape of the complex (not shown).

His-tag of the PsbH protein is linked to the N-terminal end of the polypeptide and faces the stromal side of the PSII complex. In our experiments, electron microscopy and image analysis revealed that both labeled and unlabeled PSII dimers attach preferentially with their stromal sides to the carbon support film (Fig. 5). This is in contrast with the results obtained using PSII core complexes isolated from *C. reinhardtii*, where the gold labeled complexes bind to the electron microscopy grid by the lumenal surface. This is probably one of the reasons why we were able to observe only one label per whole PSII complex compared to the results of Büchel et al. (2001) who had labeled both sides of the PSII complex.

We suppose that in our case only those particles which were tilted in respect to the carbon support film were labeled, allowing the gold particles to reach the

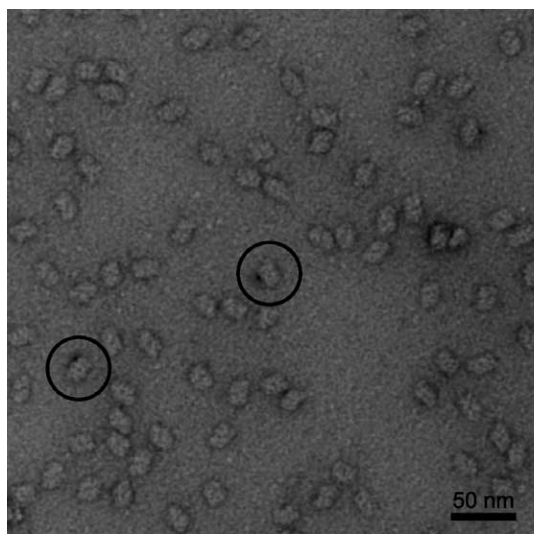

Fig. 3. Electron micrographs of dimeric PSII complexes labeled with Ni–NTA Nanogold and negatively stained with 0.75% uranyl acetate. The labeled particles are in circles.

binding His-tag site as it is demonstrated in Fig. 5B. Another reason for the lower gold-label affinity may be the fact that in cyanobacteria the N terminus of the PsbH protein is 19 amino acids shorter compared to the *C. reinhardtii*, and thus, the potential labeling site is located more inside the complex. Such location might be partially covered by neighboring amino acid side chains making the His-tag labeling site less accessible to the gold label. On the other hand, the shorter N terminus of

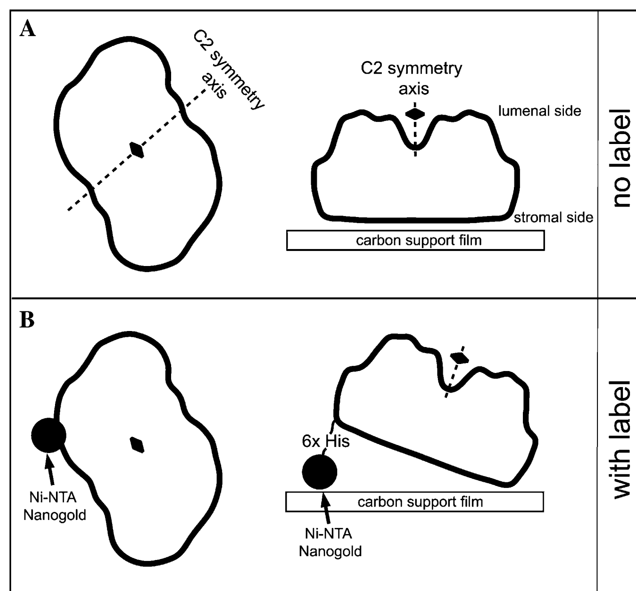

Fig. 5. Schematic representation of the preferred orientations of unlabeled (A) and labeled (B) PSII core complexes with respect to the carbon support film. The gold labels are shown as black circles. The C2 symmetry axis of PSII dimer are shown as twofold symbols and broken lines. The labeled particle is tilted in respect to the support carbon film to expose His-tag labeling site.

the *Synechocystis* PsbH protein enabled us to identify the location of the His site more precisely within the PSII complex. A careful comparison of the location of the gold clusters in *C. reinhardtii* and *Synechocystis* PSII

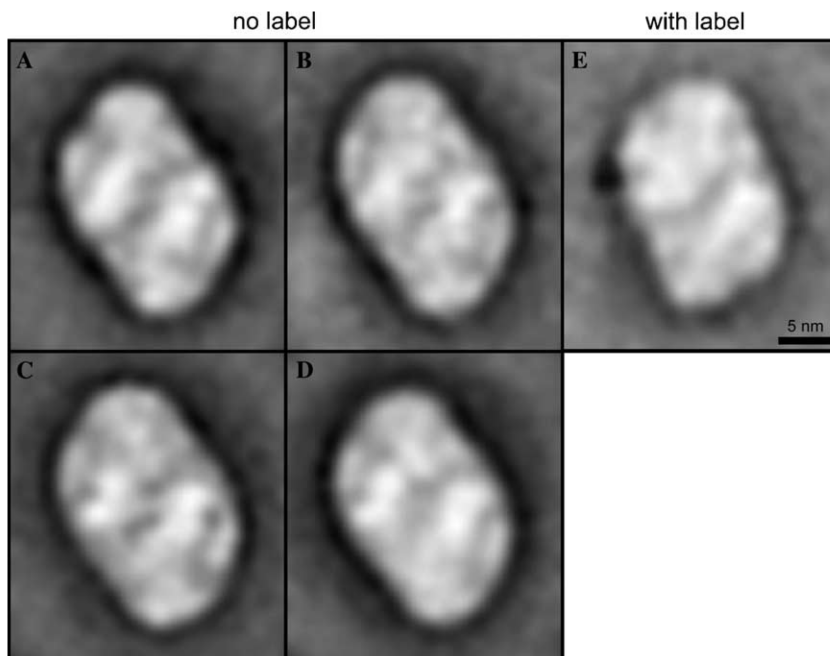

Fig. 4. Single particle analysis of top-view projections of unlabeled (A–D) and PsbH-His PSII core complex labeled with Ni–NTA Nanogold (E). The projections are presented as facing from the lumenal side of the thylakoid membrane and the number of summed images is: 530 (A), 483 (B), 385 (C), 542 (D), and 185 (E). The overall dimensions of the class average of the unlabeled PSII dimers are 23 × 16 nm, whereas the dimension of labeled PSII dimer is 23 × 17 nm.

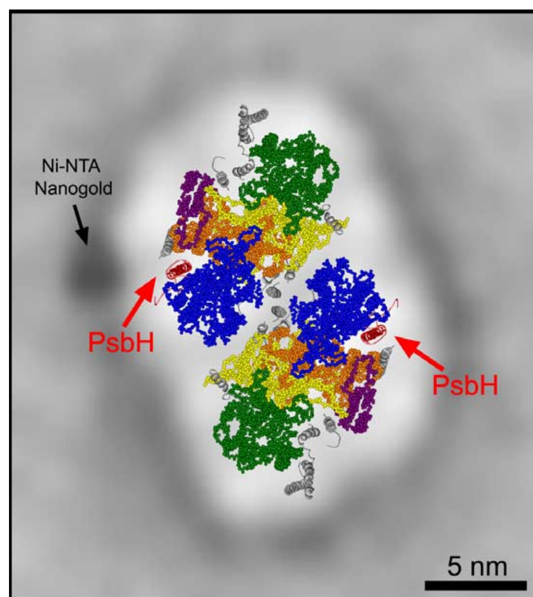

Fig. 6. Top-view projection map of the PsbH-His PSII core complex labeled with Ni–NTA Nanogold overlaid with the cyanobacterial X-ray model of the dimeric PSII core complex resolved at 3.5 Å resolution (Ferreira et al., 2004), Protein Data Bank accession number 1S5L. Carbon atoms and amino acid side chains of the major PSII subunits are in color spacefill representation; i.e. CP47 (blue), CP43 (green), D1 (yellow), D2 (orange) and cytochrome b_{559} (violet). Single transmembrane helices assigned to low molecular weight PSII subunits are represented as solid ribbons in gray color except for subunits PsbH (in red). Heteroatoms and extrinsic proteins are not shown. The Ni–NTA Nanogold label is observed only at the one side of the complex (black arrow). Since PSII particle is dimeric two PsbH subunits are present within two fold rotational symmetry around the center of the complex (red arrows).

complexes revealed that gold label in *Synechocystis* preparation is slightly shifted with respect to the longer edge of the complex.

At the present time, there are three published X-ray crystal structures of cyanobacterial PSII at, respectively, 3.8 Å (Zouni et al., 2001), 3.7 Å (Kamiya and Shen, 2003) and 3.5 Å resolution (Ferreira et al., 2004). All the three models are almost identical with respect to the location of the major PSII subunits and the arrangement of cofactors. Significant differences exist in the assignment of the single transmembrane helices representing the low molecular mass PSII subunits (Fig. 6, grey helices). In case of PsbH subunit, Zouni et al. (2001) tentatively assigned the PsbH protein to one of the three helices clustered at the contact of two monomers, while Kamiya and Shen (2003) placed the PsbH protein on the outside of the monomer close to the D2 protein. In the latest model, Ferreira et al. (2004) assigned the PsbH protein as a single transmembrane helix next to the CP47 subunit.

To locate the PsbH protein within the PSII complex we have overlaid a model of transmembrane helix organization of PSII into top-view projection map of the gold labeled PSII particle. Fig. 6 shows that Ni–NTA gold label is found to be close to the transmembrane helices of the CP47 protein. This would suggest that the PsbH protein corresponds to a single transmembrane helix in the vicinity of the CP47 protein. Although we have detected only one label attached to the PSII core complex, the observed particle is a PSII dimer and, therefore, two PsbH subunits are present within a two-fold rotational symmetry around the center of the complex (Fig. 6, red helices). The location of the PsbH subunit is in good agreement with the assignment of the PsbH subunit in the model of Ferreira et al. (2004) and it seems highly likely that single transmembrane helix close to the CP47 subunit corresponds to the PsbH protein.

Our results are also supported by the fact that the PsbH protein plays important role in the biogenesis and structure of PSII complex (Komenda et al., 2004; Suorsa et al., 2004). Deletion of the PsbH subunit in *C. reinhardtii* leads to the complete disappearance of PSII complex from the thylakoid membrane documenting its role in the stable assembly of PSII complex (O'Connor et al., 1998; Summer et al., 1997). A role of the PsbH protein in the stabilization of the PSII complex has been recently supported by recent data in which the isolated PSII complex from the *psbH* deletion mutant of *Synechocystis* 6803 was subjected to non-denaturing electrophoresis (Komenda et al., 2002). In contrast to the isolated PSII complex from the wild type, a large amount of the reaction center complex D1–D2-cyt b_{559} appeared on the gel indicating that the PsbH protein stabilizes the binding of the CP47 subunit to the D1–D2 heterodimer. Such a role could also explain the instability of the PSII core complex in *C. reinhardtii*, since weak binding of CP47 to the heterodimer could allow a fast proteolysis of PSII subunits before the complex becomes fully assembled (Komenda et al., 2003).

5. Conclusions

Ni–NTA Nanogold label consists of a gold cluster coupled to Ni^{2+}–NTA (nitrilotriacetic acid) (Hainfeld et al., 1999). The high binding affinity of Ni^{2+} to multiple histidine (His) sites was employed to label His-tagged PsbH protein. Using a mutant of *Synechocystis* 6803 with a His-tag on the N terminus of the PsbH subunit, we were able to identify the location of the PsbH subunit within the PSII complex in electron microscope. Our analysis suggests that labeling of His sites using Ni–NTA gold cluster is a powerful approach to locate specific proteins within multisubunit protein complex.

Acknowledgments

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Role of the PsbI Protein in Photosystem II Assembly and Repair in the Cyanobacterium *Synechocystis* sp. PCC 6803[1][W][OA]

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The involvement of the PsbI protein in the assembly and repair of the photosystem II (PSII) complex has been studied in the cyanobacterium *Synechocystis* sp. PCC 6803. Analysis of PSII complexes in the wild-type strain showed that the PsbI protein was present in dimeric and monomeric core complexes, core complexes lacking CP43, and in reaction center complexes containing D1, D2, and cytochrome b-559. In addition, immunoprecipitation experiments and the use of a histidine-tagged derivative of PsbI have revealed the presence in the thylakoid membrane of assembly complexes containing PsbI and either the precursor or mature forms of D1. Analysis of PSII assembly in the *psbI* deletion mutant and in strains lacking PsbI together with other PSII subunits showed that PsbI was not required for formation of PSII reaction center complexes or core complexes, although levels of unassembled D1 were reduced in its absence. However, loss of PsbI led to a dramatic destabilization of CP43 binding within monomeric and dimeric PSII core complexes. Despite the close structural relationship between D1 and PsbI in the PSII complex, PsbI turned over much slower than D1, whereas high light-induced turnover of D1 was accelerated in the absence of PsbI. Overall, our results suggest that PsbI is an early assembly partner for D1 and that it plays a functional role in stabilizing the binding of CP43 in the PSII holoenzyme.

The PSII complex is the multisubunit membrane protein complex catalyzing oxidation of water and reduction of plastoquinone in the thylakoid membranes of higher plants, algae, and cyanobacteria. The membrane-embedded core complex of PSII consists of the D1 and D2 reaction center (RC) subunits, the inner chlorophyll (Chl)-binding antenna proteins, CP47 and CP43, and a number of smaller polypeptides (for review, see Barber, 2006). D1 and D2 form a heterodimer that binds the cofactors involved in primary charge separation and subsequent electron transfer within PSII, while the main purpose of CP47 and CP43 is to deliver energy to the RC for driving electron transfer and, in the case of CP43, to help ligate the $CaMn_4$ cluster.

The roles of many of the small subunits in PSII are still unclear. In the case of PsbI, it is known to be a component of the simplest PSII unit capable of primary charge separation called D1/D2/cytochrome b-559 (Cyt b-559) or PSII RC complex (Nanba and Satoh, 1987; Webber et al., 1989). PsbI is a highly conserved PSII component showing 71% amino acid identity between the Arabidopsis (*Arabidopsis thaliana*) and *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) proteins. Recent crystal structures of cyanobacterial PSII (Ferreira et al., 2004; Loll et al., 2005) have confirmed that PsbI spans the membrane once and that it is located on the periphery of the PSII complex in close proximity to CP43 and the first and second transmembrane helices of D1, where it participates in binding a Chl molecule ($ChlZ_{D1}$).

The role of PsbI in the assembly of PSII is unclear. In plastids, the D2 protein with bound Cyt b-559 has been postulated to be the first stable assembly complex, which subsequently binds newly synthesized D1 protein to form the PSII RC complex (Müller and Eichacker, 1999). The D1 protein is synthesized as a precursor (pD1) with a carboxyl-terminal extension that is cleaved after residue Ala-344 (Takahashi et al., 1988; Nixon et al., 1992) by a specific processing endoprotease, CtpA (Anbudurai et al., 1994). In higher plants, the extension usually consists of nine residues and it is removed in a single step. In contrast, the 16-amino acid C-terminal extension of D1 in *Synechocystis* 6803 is cleaved in two steps (Inagaki et al., 2001). The primary cleavage occurs after Ala-352, resulting in formation of a processing intermediate termed iD1 (Komenda et al., 2007), which in *Synechocystis* 6803 is mainly associated with RC complexes (Komenda et al., 2004).

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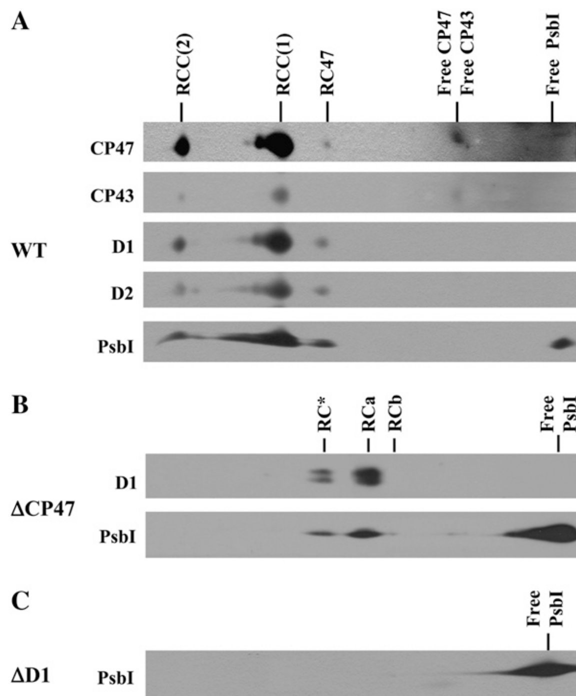

Figure 1. Immunoblots of thylakoid membrane proteins from *Synechocystis* 6803 wild type (WT; A), *psbB* deletion mutant ΔCP47 (B), and *psbA* deletion mutant ΔD1 (C) after their separation by 2D BN/SDS-PAGE. Thylakoid proteins were separated by 2D BN-PAGE, blotted onto PVDF membrane, and immunodecorated using antibodies raised against D1, D2, CP47, CP43, and PsbI. Designation of complexes: RCC(2) and RCC(1), dimeric and monomeric PSII core complexes, respectively; RC47, PSII core complex lacking CP43; RC*, RCa, and RCb, RC complexes. One microgram of Chl was loaded for each sample.

In contrast to Cyt b-559, PsbI is not critical for assembly of PSII. A mutant of *Synechocystis* 6803 lacking the *psbI* gene was able to grow photoautotrophically and its PSII oxygen evolution activity was 70% to 75% of wild-type levels (Ikeuchi et al., 1995). In *Chlamydomonas reinhardtii*, inactivation of the *psbI* gene decreased oxygen evolution activity and D1 content in the resulting photoautotrophic mutant to 10% to 20% of wild-type levels (Künstner et al., 1995). A *psbI* deletion mutant of tobacco (*Nicotiana tabacum*) contained reduced amounts of dimeric PSII and PSII light-harvesting complex II supercomplexes. The tobacco mutant also exhibited a modified primary quinone acceptor, Q_A, an increased sensitivity to high light, and no phosphorylation of the RC proteins, D1 and D2 (Schwenkert et al., 2006). In summary, inactivation of *psbI* causes a decrease in PSII photochemical activity and content in all organisms studied to date, but the underlying molecular basis for this effect remains unknown.

Here we present a detailed analysis of the role of PsbI during assembly and repair of cyanobacterial PSII. The results showed that although the presence of PsbI is not critical for PSII biogenesis, the protein is important for stable incorporation of CP43 into PSII and possibly for stabilizing newly synthesized D1 protein.

RESULTS

Identification of the PsbI Protein in PSII Complexes and Early Assembly Intermediates

To investigate the role of PsbI in PSII assembly, we first screened for the presence of PsbI in various types of PSII complexes and PSII assembly intermediates. An excellent tool for this purpose is the two-dimensional (2D) separation of membrane proteins consisting of blue native (BN) PAGE in one direction and denaturing PAGE in the second direction (2D BN/SDS-PAGE) followed by immunoblotting. In this way, we probed for the presence of PsbI in PSII complexes of wild type and several mutant strains of *Synechocystis* 6803 blocked at a particular step in assembly.

In wild type, the majority of the large PSII protein subunits CP47, D2, and D1 accumulate in monomeric [RCC(1)] and dimeric [RCC(2)] core complexes as well as in a core subcomplex lacking CP43 (RC47; see also Komenda et al., 2004). PsbI was detected in all these complexes and, in addition, was found in the low-molecular-weight region, probably in an unassembled form (Fig. 1A).

To investigate at what stage PsbI bound to PSII during assembly, we tested for its presence in the PSII complexes found in strain ΔCP47, which is unable to synthesize CP47 and so is unable to assemble PSII beyond formation of a PSII RC complex (Komenda et al., 2004). In this strain, native BN-PAGE resolved three RC complexes with different mobilities. The precise subunit composition of each complex is unclear, but each contained D2, both subunits of Cyt b-559, and either D1 or its processing intermediate, iD1. The most abundant RC complex was RCa, followed by RC*, with a mobility similar to that of RC47, while the smallest RCb complex was present in very low amounts. Immunoblots confirmed the presence of PsbI in both RC* and RCa, although the majority of the protein was detected in the low M_r region as a free protein (Fig. 1B). The low abundance of RCb in the sample analyzed in Figure 1B did not allow a reliable evaluation of the presence of PsbI. However, PsbI could be detected in this particular complex in frozen thylakoids of ΔCP47 stored for a longer time, indicating possible artefactual origin of RCb.

In contrast, in a *psbA* deletion strain (ΔD1) in which CP47, CP43, and D2 are synthesized but do not assemble into a larger complexes (Komenda et al., 2004), the PsbI protein was found exclusively as a free protein (Fig. 1C).

The overall level of PsbI in the ΔD1 mutant was much lower than in wild type, but after additional inactivation of the *slr0228* gene encoding an FtsH protease homolog, the amount of PsbI reached wild-type levels (Supplemental Fig. S1). This observation

suggested that unassembled PsbI is removed from the thylakoid membrane through the action of FtsH (slr0228) as observed previously for unassembled D2 and CP47 (Komenda et al., 2006).

Absence of PsbI Destabilizes Binding of CP43 in the PSII Core Complex But Does Not Inhibit Assembly of the PSII RC Complex

To characterize the role of PsbI in the assembly of PSII core and RC complexes, we constructed a *psbI* deletion mutant (ΔPsbI) in which the *psbI* gene was replaced by a zeocine resistance cassette. In agreement with earlier data (Ikeuchi et al., 1995), the resulting mutant exhibited a somewhat slower rate of photoautotrophic growth and its PSII photochemical activity, measured as the variable fluorescence yield or the light-saturated rate of oxygen evolution, was lower by 30% to 40% in comparison with wild type (Supplemental Table S1). On the other hand, electron transfer between the plastoquinone electron acceptors, Q_A and Q_B, was similar in wild type and mutant (data not shown).

To characterize PSII assembly in wild type and ΔPsbI, we pulse labeled cells with [^{35}S]Met/Cys at 23°C and analyzed the incorporation of radiolabel into PSII subunits following 2D BN/SDS-PAGE to resolve the different PSII complexes. By performing the experiment at 23°C we hoped to slow assembly down so that assembly intermediates could be more easily detected.

In wild type, 80% to 90% of the D1 subunit was found in the dimeric and monomeric core complexes, RCC(2) and RCC(1), as assessed by the intensity of the Coomassie Blue-stained band, with the remaining 10% to 20% in the RC47 complex (Fig. 2A; Supplemental Table S2). In striking contrast, in ΔPsbI, only 20% and 30% of D1 was present in dimeric and monomeric PSII core complexes, respectively. Instead, a significant level of D1 was found in PSII complexes lacking the full complement of CP43: 20% was detected in the dimeric core complex lacking one copy of CP43 (RCC-RC47), 5% in the dimeric core complex lacking both CP43 copies [RC47(2)], and 25% in the monomeric RC47 (Fig. 2B; Supplemental Table S2). Thus, binding of CP43 was inhibited or destabilized in about 50% of the PSII core complexes in ΔPsbI.

Autoradiograms obtained from the same gels were used to assess the incorporation of radioactive label into the various PSII proteins. For wild type, the D1 protein was preferentially labeled in all PSII complexes, including the RCa complex, as expected because of selective D1 replacement during PSII repair. However, 50% to 60% of labeled D1, including its two incompletely processed forms, pD1 and iD1, was found in the fraction of small complexes and free unassembled proteins (Fig. 2C, U.P.). This unassembled D1 fraction can be detected in wild type only by a short radioactive pulse at a lower temperature (23°C), while at the growth temperature (30°C), the assembly of D1 into PSII complexes is so fast that free D1 forms are not detectable (see Komenda et al., 2006). In the unassembled fraction, pD1 migrated in two bands under native conditions in the first dimension, with the mobility of the larger one (Fig. 2C, arrow) comigrating with a weak band in the PsbI immunoblot (Fig. 2G, arrow). The data suggested the existence of a putative pD1-PsbI complex (see below). When the label was chased for 20 min in the presence of the protein synthesis inhibitor, chloramphenicol (CAP), the labeled D1 protein found in RCa and in the free protein fraction disappeared, and labeling of the mature D1 subunit increased 3.5 times in RCC(1) and 7 times in RCC(2) (Fig. 2E; Supplemental Table S2). This result confirmed that unassembled D1 and the RCa complex were true assembly intermediates and not dead end products.

The D1 protein was also preferentially labeled in the ΔPsbI mutant, but its distribution among the various complexes differed from wild type. After the pulse, 60% of the labeled D1 protein was present in the RC47 complex and no labeled D1 was observed in either RCa or the unassembled protein fraction (Fig. 2D). During the chase, 80% of the labeled D1 disappeared from RC47, but only 20% appeared in PSII complexes larger than RC47 (Fig. 2F; Supplemental Table S2). This result indicated that most of the newly synthesized D1 protein incorporated into RC47 complexes was degraded before the complex could be assembled further into monomeric and dimeric PSII core complexes.

We also constructed a series of double mutants in which the deletion of the *psbI* gene was performed in strains lacking the large PSII subunits CP43, CP47, or D2. In the strain lacking CP43 (ΔCP43), PSII assembly could not progress beyond formation of the RC47 complex and the strongly radiolabeled D1 protein almost exclusively accumulated in this complex (Supplemental Fig. S2, A and C). A similar pattern was also maintained after additional removal of PsbI, except that there was a slight increase in the amount of labeled D2 in the RC47 complex and in the unassembled protein fraction (Supplemental Fig. S2, B and D). The very similar intensity of radiolabeled D1 band in the RC47 complex indicated similar turnover rates of D1 in both strains.

The *psbB* deletion strain ΔCP47 has already been shown to accumulate small amounts of the RC complexes RC*, RCa, and RCb, and all these complexes contained PsbI (Fig. 1B). To find out whether their formation was dependent on the presence of PsbI, we also constructed and characterized the double mutant ΔCP47/ΔPsbI. On Coomassie Blue-stained gels of radioactively labeled thylakoid proteins separated by 2D BN/SDS-PAGE, only the free unassembled CP43 was visible in both ΔCP47 and ΔCP47/ΔPsbI strains (Fig. 3, A and B). The formation of RC complexes in both strains was confirmed by autoradiography (Fig. 3, C and D), although the amount of RC* and RCb complexes in the PsbI-less mutant was increased at the

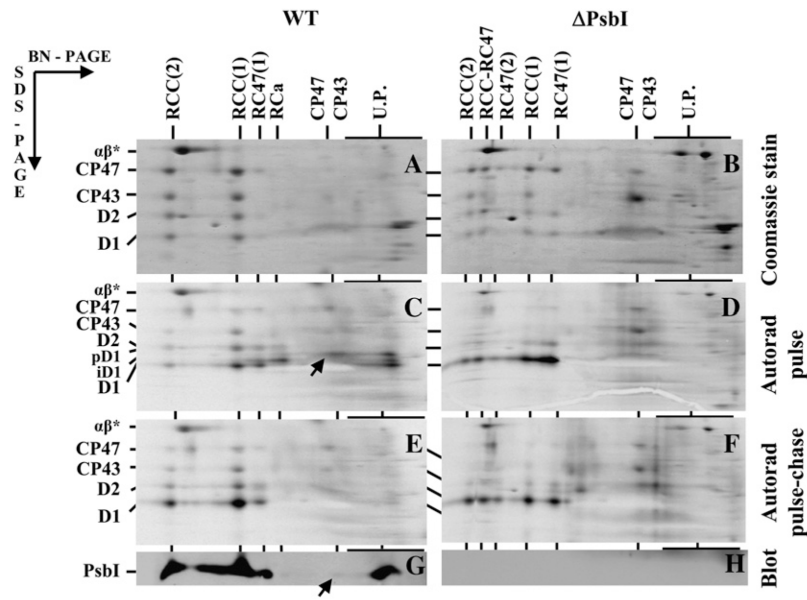

Figure 2. Pulse-chase analysis of wild type and the *psbI* deletion strain ΔPsbI. Cells of wild type (A, C, E, and G) and ΔPsbI (B, D, F, and H) were radiolabeled at 500 μmol photons m^{-2} s^{-1} and 23°C with a mixture of [^{35}S]Met/Cys for 10 min (pulse) and then CAP (1 mg mL^{-1}) was added and cells incubated at the same temperature at 125 μmol photons m^{-2} s^{-1} for another 20 min (chase). Labeled cells were used for isolation of thylakoids, which were analyzed by 2D BN/SDS-PAGE. A and B, Coomassie Blue-stained gels of proteins after the pulse. C and D, Autoradiograms of the same samples with the putative D1-PsbI complex designated by arrow in C. E and F, Autoradiograms of proteins after pulse chase. G and H, Low-M_r region with PsbI detected by immunoblotting (blot) and putative D1-PsbI complex designated by an arrow. Designations of proteins are as described in the legend to Figure 1. RCC-RC47 designates dimeric PSII core complex lacking only one CP43 copy and RC47(2) the dimeric PSII core lacking both CP43 copies. U.P., Unassembled proteins. α- and β-subunits of ATP synthase (designated by $\alpha\beta$*) were used as internal standards during quantification of D1-stained and -labeled bands (see Supplemental Table S2). The arrows in C and G show the complex of PsbI and pD1 in wild type. Six micrograms of Chl was loaded for each sample.

expense of RCa. The autoradiogram of ΔCP47 also indicated the presence of a putative pD1-PsbI complex (Fig. 3C, arrow) previously found in wild type labeled at 23°C (Fig. 2C). Quantification of D1 by immunoblotting (Supplemental Fig. S3) revealed a slightly lower amount of mature D1 and partially processed iD1 (Komenda et al., 2007) in the double mutant. Overall, these data show that formation of the RC complexes was not dependent on the presence of PsbI.

In the Absence of D2, the Majority of the D1 Protein Is Associated with PsbI

To confirm that PsbI was able to form a complex with D1, experiments were conducted using a *psbEFLJ* deletion strain, which is unable to synthesize Cyt b-559 and, as a consequence, is also unable to accumulate the D2 protein (Komenda et al., 2004). In this strain (ΔCYT), most of PsbI was present as a free protein, but

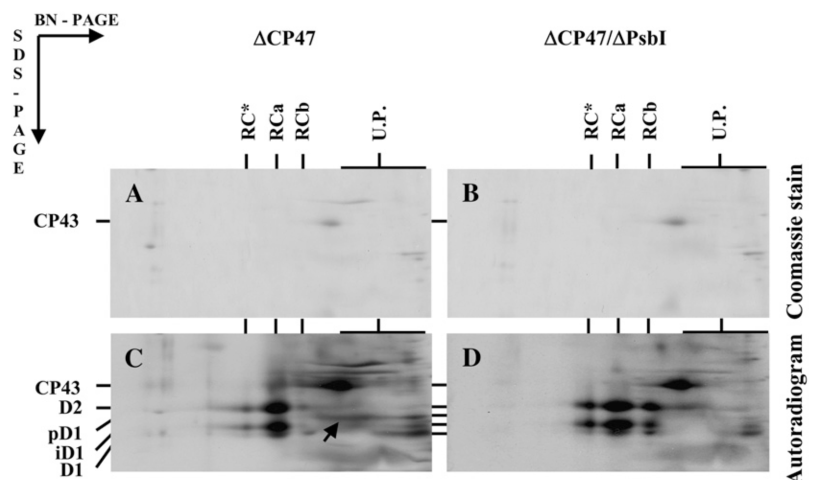

Figure 3. Analysis of thylakoid membrane proteins in the *psbB* deletion mutant ΔCP47 and the double deletion mutant ΔCP47/ΔPsbI. Cells of the *Synechocystis* 6803 strains were radiolabeled at 500 μmol photons m^{-2} s^{-1} and 29°C with [^{35}S]Met/Cys for 30 min and their thylakoid proteins were separated by 2D BN/SDS-PAGE. Designations of proteins are as described in the legend to Figure 1; the arrow indicates the complex of PsbI and pD1. Six micrograms of Chl was loaded for each sample.

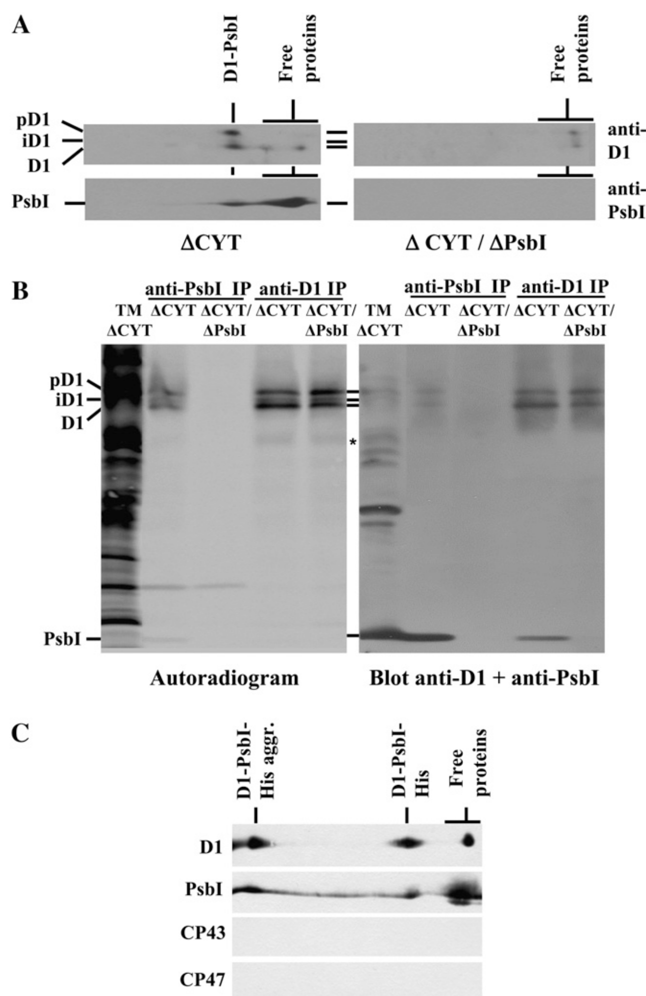

Figure 4. Identification of a D1-PsbI precomplex. A, Thylakoid membrane proteins (1 μg of Chl) of the ΔCYT and ΔCYT/ΔPsbI strains were separated by 2D BN/SDS-PAGE, transferred onto PVDF membrane, and detected by antibodies against D1 and PsbI. B, Pulse-labeled thylakoid proteins (5 μg of Chl) from the ΔCYT and ΔCYT/ΔPsbI strains were immunoprecipitated using antibodies specific for D1 (anti-D1 IP) or PsbI (anti-PsbI IP) and the immunoprecipitates together with thylakoids from ΔCYT (TM) were analyzed by SDS-PAGE, blotted onto PVDF membrane, autoradiographed (left, autoradiogram), and then probed with antibodies against both PsbI and D1 proteins (right, blot anti-D1 + anti-PsbI). *, A putative 23-kD D1 synthesis intermediate. C, Immunoblots of D1, PsbI, CP43, and CP47 after 2D BN/SDS-PAGE of the protein fraction bound to nickel-affinity column loaded with solubilized thylakoids of the PsbI-His/ΔPsbI/ΔCYT strain. D1-PsbI-His aggr., Aggregates of D1 and PsbI-His.

there was an additional small PsbI complex (Fig. 4A) similar in size to the putative pD1-PsbI complex detected in wild type (Fig. 2G, arrow). Indeed, when the 2D blot of the ΔCYT strain was reprobed with an antibody against D1, each D1 form (i.e. pD1, iD1, and D1) migrated in two bands under native conditions (see also Komenda et al., 2004). About 80% of the protein was present in the larger complex with the same mobility as the small PsbI-containing complex (Fig. 4A). When a strain lacking both the *psbEFLJ* operon and the *psbI* gene was analyzed (ΔCYT/ΔPsbI), the larger D1 band was no longer detected and the overall level of D1 decreased to 25% of that found in ΔCYT (see Fig. 4A). The identical D1-PsbI complex was also detected in the ΔCP47 strain (Fig. 3, arrow) but not in the ΔCP47/ΔPsbI double mutant. Overall, these results suggested that in the absence of D2, the majority of D1 was associated with PsbI. This association appeared to stabilize unassembled D1 and enabled its higher accumulation in the thylakoid membrane.

To strengthen these conclusions, we performed immunoprecipitation experiments using antibodies against PsbI and D1 and radiolabeled thylakoids isolated from the ΔCYT and ΔCYT/ΔPsbI strains. In ΔCYT, both antibodies precipitated PsbI together with all forms of D1 but not the other PSII proteins (Fig. 4B). For the ΔCYT/ΔPsbI strain, we were unable to immunoprecipitate D1 using the antibody against PsbI, while the antibody against D1 precipitated only the D1 protein. These results confirmed the existence of the D1-PsbI complex in the ΔCYT strain. The presence of labeled pD1 in the immunoprecipitate further indicated that binding of PsbI occurred soon after or during synthesis of D1.

The third line of evidence for binding of PsbI to D1 was obtained using a strain lacking the *psbEFLJ* operon in which the original *psbI* gene was replaced by a His-tagged copy. Thylakoids from the PsbI-His/ΔPsbI/ΔCYT strain were solubilized with dodecyl-maltoside (DM) and the extract was loaded on the nickel-affinity chromatography column. Bound proteins were analyzed by 2D BN/SDS-PAGE and immunoblotting using antibodies against PsbI, D1, CP43, and CP47. The majority of PsbI-His was present as free protein but a substantial portion was found in a small complex with D1 (Fig. 4C). Neither CP43 nor CP47 were found in the eluate, confirming specific binding of PsbI-His to D1.

PsbI Does Not Undergo Fast Turnover But Its Absence Accelerates Turnover of the D1 Protein

Given that PsbI binds to D1 in the PSII complex (Ferreira et al., 2004; Loll et al., 2005), we were interested to learn whether both PsbI and D1 were turned over during PSII repair or whether PsbI was stable and reused during D1 replacement. Analysis of thylakoids isolated from wild-type cells that had been exposed to high light for 4 h in the presence of the protein synthesis inhibitor lincomycin showed that PsbI is much more stable than D1 (Fig. 5). Under these illumination conditions, conducted in the absence of protein synthesis, the majority of D1 and D2 was degraded, while other proteins like CP47, CP43, and PsbI were released from photodamaged PSII and were detected in the unassembled protein fraction. Pulse-chase experiments (Fig. 6, bottom sections) also indicated that a weakly labeled band corresponding to PsbI remained stable over the period of the chase.

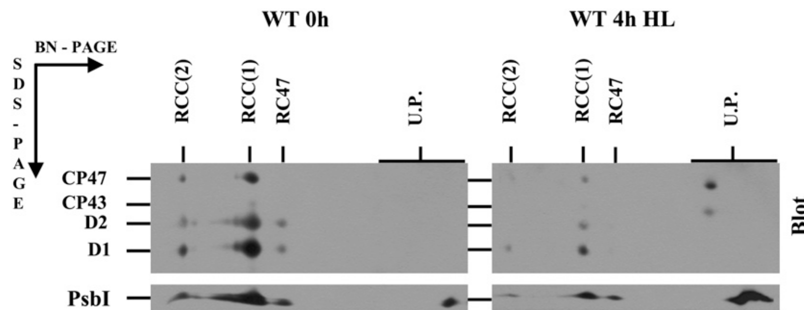

Figure 5. Immunoblots of thylakoid membrane proteins from the *Synechocystis* 6803 wild-type strain before and after 4-h exposure to high light. Cells of wild type were exposed to high irradiance (1,000 μmol photons m^{-2} s^{-1}) for 4 h in the presence of the protein synthesis inhibitor lincomycin (100 μg mL^{-1}). Thylakoid membrane proteins (1 μg of Chl) were separated by 2D BN/SDS-PAGE, transferred onto PVDF membrane, and probed with antibodies against the D1, D2, CP43, CP47, and PsbI proteins. Designations of proteins are as described in the legend to Figure 1. To allow direct comparison of protein bands, thylakoids from control and photoinhibited cells were analyzed on a single gel and blot.

Although the PsbI protein is relatively stable, the turnover of D1 is significantly accelerated in its absence in ΔPsbI. Pulse-chase labeling revealed a half-life of D1 in wild type of about 2 h, while in the mutant it was reduced to approximately 30 min (Fig. 6, top). To find out if this high rate of D1 turnover in the mutant was related to a higher sensitivity of PSII photochemistry to photodamage, we also evaluated the time course of light-induced inhibition of oxygen evolution in wild-type and mutant cultures subjected to white light of 500 μmol photons m^{-2} s^{-1} either in the absence or presence of lincomycin. Cultures of wild type and ΔPsbI treated with lincomycin did not exhibit any differences in the decline of oxygen evolution (Fig. 7A, right), while in the absence of lincomycin, the decrease in activity in ΔPsbI was somewhat faster than in wild type (Fig. 7A, left). Recovery from photoinhibition under low-light conditions was also slightly slower in ΔPsbI as evidenced by assessment of oxygen evolution in photoinhibited cells of wild type and ΔPsbI during subsequent incubation at 50 μmol photons m^{-2} s^{-1} (Fig. 7B). The results showed that the oxygen-evolving PSII complexes of the mutant are equally sensitive to light-induced inactivation as complexes of wild type, while the regeneration of active PSII is less efficient despite the accelerated turnover of D1.

DISCUSSION

Role of PsbI in the Formation of the PSII Core and RC Complexes

Although the PsbI protein is known to be a component of the isolated PSII RC complex (Ikeuchi et al., 1989; Webber et al., 1989), it has been unclear at what stage PsbI actually binds to PSII during assembly. Here we show that PsbI binds to PSII at an early stage. In particular, we have shown that PsbI is a component of the three RC complexes found in the ΔCP47 strain. All three complexes lack CP47 and CP43 and contain D1, D2, Cyt b-559, and PsbI and so resemble the isolated PSII RC complex in composition. Given that the complexes show different electrophoretic mobilities, it is possible that they differ with respect to the presence of additional assembly factors not found in the final PSII complex. These proteins remain unknown but the modified stoichiometry of these RC complexes induced by the absence of PsbI suggests that PsbI might affect binding of these proteins. Despite binding to PSII early in assembly it is clear, however, that PsbI is not crucial for accumulation of RC complexes.

In contrast, there is a significant stabilization effect of PsbI on the binding of CP43 within the PSII core complex (Fig. 8, step 4), in excellent agreement with the close proximity of PsbI and CP43 in the recent structural studies models of PSII (Ferreira et al., 2004; Loll et al., 2005). The increased levels of RC47 complexes in the PsbI mutant also help to explain the reduced oxygen-evolving activity displayed by the mutant.

Relationship between D1 and PsbI during de Novo Assembly of PSII

Our analysis of the ΔCYT and ΔD1 strains unable to form the PSII RC complex allowed us to look at assembly steps that precede the formation of the RC complex. One of these steps is the formation of a D2-Cyt b-559 precomplex (Fig. 8, step 1B) originally observed in plant etioplasts (Müller and Eichacker, 1999). In this study, we observed the formation of another RC precomplex consisting of D1 and PsbI (Fig. 8, step 1A). Support for the existence of this specific precomplex was based on three lines of evidence: (1) identification of D1-PsbI complexes in thylakoids of wild type, ΔCP47, and ΔCYT by 2D analysis; (2) coimmunoprecipitation of PsbI and D1 from the ΔCYT strain; and (3) copurification of D1 with PsbI-His. The first approach also showed that removal of PsbI led to a decrease in the level of unassembled D1 protein in wild type (Fig. 2, C [arrow] and D), in the CP47-less

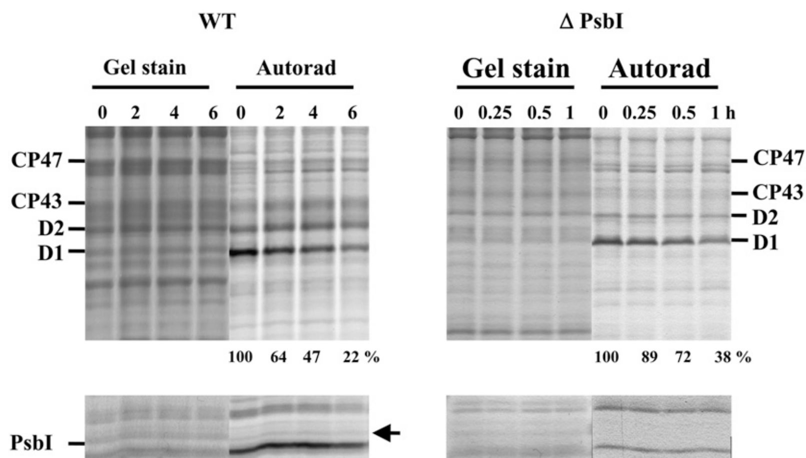

Figure 6. Degradation of the PSII proteins in the wild-type and ΔPsbI strains under high irradiance monitored by radioactive pulse-chase labeling. Cells of both strains were subjected to 250 μmol photons m^{-2} s^{-1} of white light for 20 min in the presence of [^{35}S]Met/Cys. Then the cells were washed, supplemented with unlabeled Met/Cys, and subjected to 500 μmol photons m^{-2} s^{-1} of white light for 6 h. Thylakoids were isolated, analyzed by SDS-PAGE, the gel was stained (Gel stain), and the radioactive labeling of the proteins was visualized using a PhosphorImager (Autorad). Quantification of radioactivity in the D1 band was performed by ImageQuant software with samples of each strain equally loaded on Chl basis (2 μg of Chl; see Gel stain) in a single gel. The radioactivity incorporated into the D1 band of each strain during pulse was taken as 100%; numbers show means of three measurements; SD did not exceed 7%. The low-M_r region of the gel is shown in the bottom sections and the stable band of PsbI is designated by an arrow.

strain (Fig. 3C), and in the ΔCYT strain (Fig. 4A), suggesting an important role of PsbI in D1 stabilization before its incorporation into PSII. In ΔCYT, which lacks D2 due to effects of deleting the *psbEFLJ* operon, PsbI could be coimmunoprecipitated not only with mature D1 but also the unprocessed forms, pD1 and iD1, indicating an early interaction of PsbI with D1 even before its maturation. In this respect, it is interesting to note that the antibodies against PsbI and D1 also precipitated the 23-kD N-terminal part of D1 (Fig. 4B, designated by *). This D1-related species seems to be a D1 synthesis intermediate as it was not precipitated by antibody against the C terminus of D1 and disappears during the chase. This finding raises the interesting possibility that binding of PsbI to D1 occurs during D1 synthesis on membrane-bound ribosomes. In agreement with this idea, a significant level of PsbI was found in membrane-bound polysomes (data not

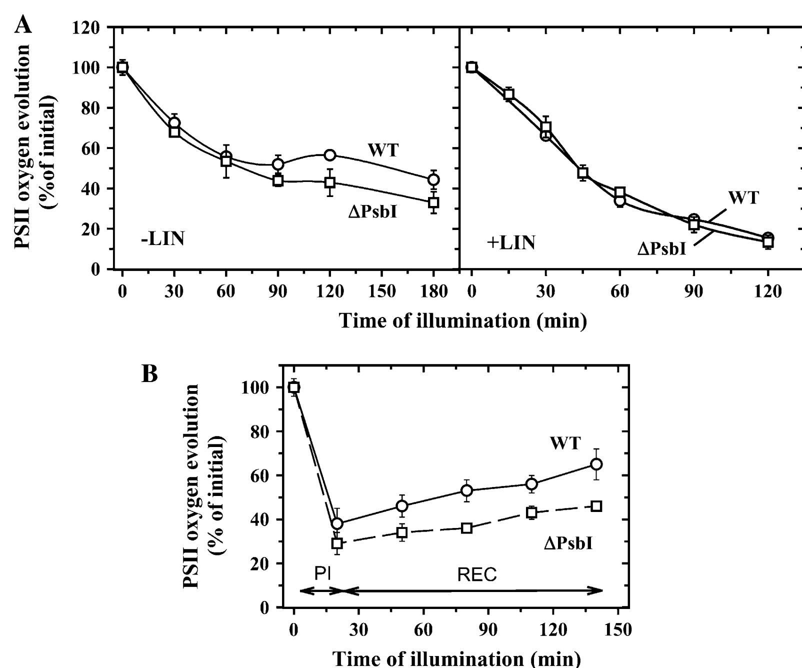

Figure 7. PSII repair under high and low irradiance in cells of wild type and ΔPsbI. A, Cells of wild type (circles) and ΔPsbI (squares) were illuminated with 500 μmol photons m^{-2} s^{-1} of white light for 180 min in the absence (left) or for 120 min in the presence (right) of 100 μg mL^{-1} lincomycin and during illumination PSII oxygen-evolving activity was assayed in whole cells. B, Cells of wild type (circles) and ΔPsbI (squares) were illuminated at 2,000 μmol photons m^{-2} s^{-1} for 20 min (PI), then the cells were transferred to low irradiance of 50 μmol photons m^{-2} s^{-1} and incubated for an additional 120 min (REC). During these light regimes, PSII oxygen-evolving activity was assayed in whole cells. Values in the plots represent means of three measurements ± SD. Initial values for wild type and ΔPsbI were in the range of 372 ± 27 and 258 ± 13 μmol O_2 mg Chl^{-1} h^{-1}, respectively.

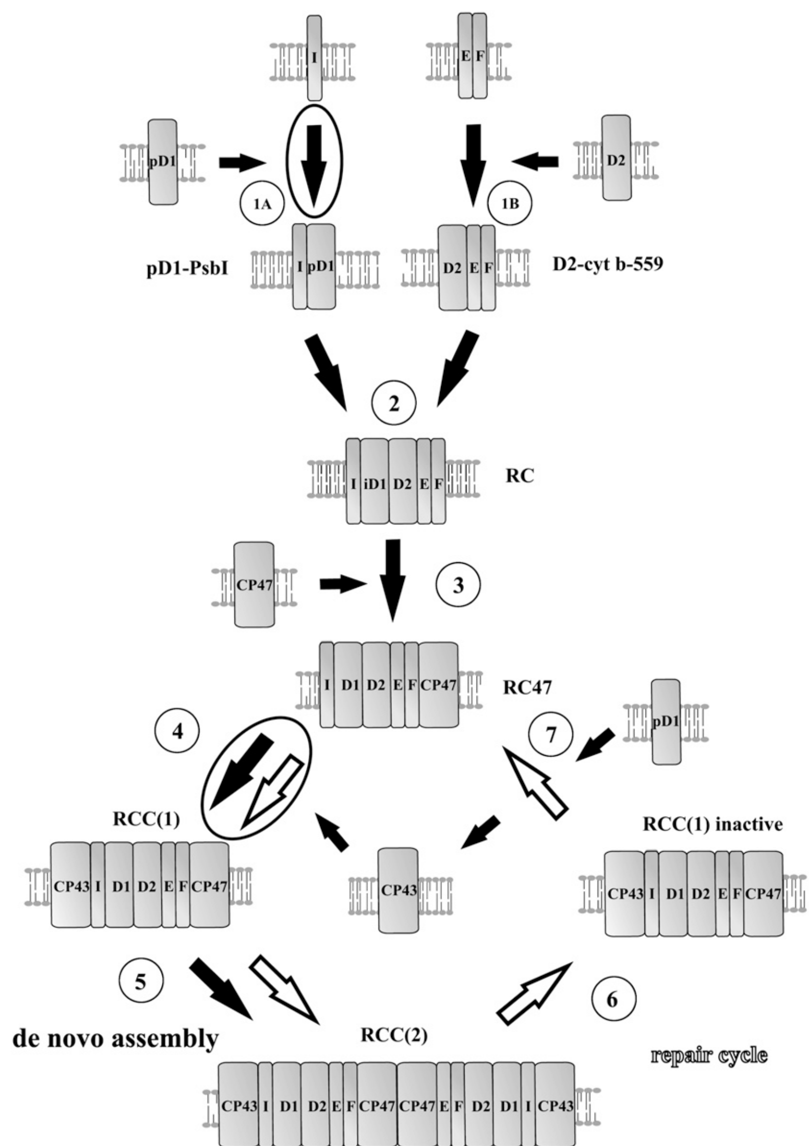

Figure 8. Illustration of function of PsbI during PSII assembly and repair. De novo assembly of PSII (large black arrows) starts with association of PsbI (I) with pD1 (step 1A) forming pD1-PsbI precomplex on one side, and association of Cyt b-559 protein subunits α and β (E and F) with the D2 protein forming Cyt b-559-D2 precomplex on the other side (step 1B). Assembly continues by formation of the RC complex from both precomplexes (step 2). Then CP47 (step 3) and CP43 (step 4) are attached and resulting PSII core monomers RCC(1) form the PSII core dimer [RCC(2), step 5]. PSII repair cycle (white arrows) starts with inactivation of the D1 protein and monomerization (step 6). Then it continues by detachment of CP43 and selective replacement of the D1 protein (step 7). The final two steps, the CP43 attachment and PSII dimerization, seem to be common with the de novo assembly pathway. PsbI is important for processes of formation of pD1-PsbI precomplex (step 1A) and attachment of CP43 to RC47 (step 4) as indicated by encircled arrows. For simplicity, other small and extrinsic PSII subunits were omitted. pD1, Unprocessed forms of D1; iD1, partially processed forms of D1.

shown). We therefore assume that PsbI may stabilize the D1 nascent chain before it is incorporated into the de novo assembled PSII.

2D analysis of wild-type thylakoids showed a fraction of PsbI that is not assembled in any PSII complex. This fraction cannot originate from PSII disassembly or from release of PsbI from complexes during native electrophoresis, as no unassembled D1 and D2 are found in wild type at growth temperature and unassembled PsbI is also found in the strain lacking the D1 protein, the regular binding partner of PsbI (Fig. 1C). In this respect, PsbI is reminiscent of the α-subunit of Cyt b-559. A significant fraction of this protein also remains unassembled in wild-type thylakoids (Komenda et al., 2004). It is possible that small stabilizing partners of D1 and D2 like PsbI, PsbE, or PsbF exist in a surplus nonstoichiometric number of copies in comparison with D1 and D2. In this way, the membrane might be prepared for the prompt initiation of de novo assembly of PSII under variable environmental conditions that require a fast response by the organism.

Relationship between D1 and PsbI during the Repair of PSII

Despite the close structural relationship between D1 and PsbI, their half-lives are very different. The D1 protein is known as a fast turning-over protein, while PsbI is much more stable. Because D1 turnover is controlled by the same FtsH (*slr0228*) protease as the free pool of PsbI (Supplemental Fig. S1), D1 turnover must be tightly regulated to prevent parallel degradation of PsbI. On the other hand, when PsbI is absent in the *psbI* deletion mutant, D1 degradation is accelerated in comparison with wild type even though the effectiveness of PSII repair in the mutant is lower. The accelerated D1 turnover seen in the PsbI mutant is most probably related to the destabilization of CP43

binding to PSII in the absence of PsbI. The 2D analysis of the pulse-chase labeled ΔPsbI strain showed that fast D1 replacement occurs in the RC47 complex, as observed for the wild type (Komenda et al., 2006), but that RC47 complexes containing the newly replaced D1 were impaired in the rebinding of CP43 to form active core complexes and instead underwent further cycles of D1 degradation and reinsertion. The resulting frequent but futile replacement of D1 would represent a significant energy demand for the organism that would be another reason for the slower photoautotrophic growth of the mutant in comparison with wild type. On the other hand, the efficient insertion of the D1 protein into RC47 of the ΔPsbI and ΔCP43/ΔPsbI strains indicate that the PsbI protein is not involved in insertion of the newly synthesized D1 protein during selective D1 replacement.

MATERIALS AND METHODS

Construction and Cultivation of Cyanobacterial Strains

The strains used in the study were derived from the Glc-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams, 1988) referred to as wild type. The following, previously described strains were used in the study: (1) the CP43-less strain ΔCP43 with the *psbC* gene inactivated by kanamycin resistance cassette (Vermaas et al., 1988); (2) the CP47-less strain ΔCP47 with the *psbB* gene inactivated by spectinomycin resistance cassette (Eaton-Rye and Vermaas, 1991); (3) the ΔCYT strain with the *psbEFLJ* operon replaced by kanamycin resistance cassette (Pakrasi et al., 1988); and (4) the D1-less strain ΔD1 with *psbA1*, *psbA2*, and *psbA3* genes inactivated by CAP, kanamycin, and spectinomycin resistance cassettes (Tichý et al., 2003).

The ΔPsbI strain was prepared by replacement of most of the *psbI* gene (nucleotides 6–105) by a zeocine resistance cassette using megaprimer PCR method (Burke and Barik, 2003). We have adapted this method to generate linear deletion constructs in vitro containing upstream and downstream regions of the *psbI* gene with the zeocine resistance cassette in the middle. In the first step, upstream and downstream regions of *psbI* were separately amplified using long fusion primers recognizing in one direction the *psbI* gene and in the other direction the zeocine cassette: 5′-ACATTAATTGCGTTGCG CTCACTGCTTAACATAAATTCTCCTTAG-3′ and 5′-CAACTTAATCGCCTTGCAGCACATCGACTTTGAATAAGCTTTAGC-3′(the *psbI* part is underlined). These fusion primers were used in pairs with *psbI* forward and reverse primers: 5′-GGTAATTCTCGATTCAGTTG-3′ and 5′-GGTGTGATCAAATACTCCTG-3′. In the second step, the zeocine resistance cassette (*Streptoalloteichus hindustanus*, Invitrogen) was amplified using PCR products from the first step as primers. Finally, the complete deletion construct was amplified using *psbI* forward and reverse primers and used for transformation of *Synechocystis* 6803 cells. Transformants were selected and segregated on zeocine-containing agar plates; their full segregation was confirmed by PCR.

Multiple *psbI* deletion strains were obtained by transformation of single mutants lacking *psbC*, *psbB*, *psbEFLJ*, and *psbA* genes using chromosomal DNA from ΔPsbI and their selection for the additional resistance to zeocine.

The PsbI-His strain expressing PsbI tagged with the 6× His tag at its N terminus under control of the *psbA2* promoter (PsbI-His/ΔPsbI) was constructed using the *pSBA* plasmid (Lagarde et al., 2000) in a procedure analogous to that described in Tichý et al. (2003). The resulting strain synthesizing both wild-type and His-tagged forms of PsbI was transformed with chromosomal DNA from the *psbI* deletion mutant and selected for the resistance to zeocine. The complete deletion of the *psbI* wild-type copies in the PsbI-His strain was confirmed by PCR. The strain PsbI-His/ΔPsbI/ΔCYT was obtained by transformation of PsbI-His/ΔPsbI using chromosomal DNA from the *psbEFLJ* deletion strain and selection for the additional resistance to kanamycin.

Liquid cultures were grown in 100 to 200 mL BG11 using 500-mL conical flasks, aerated using an orbital shaker, irradiated with 30 μmol photons m^{-2} s^{-1} of white light at 29°C, and were used when they reached a Chl concentration of about 5 μg mL^{-1}. Solid medium contained in addition 10 mM Tes/NaOH, pH 8.2, 1.5% agar, and 0.3% sodium thiosulphate (Pakrasi et al., 1988). Media for cultivation of nonautotrophic strains contained in addition 5 mM Glc.

Photoinhibition experiments were performed with cells cultivated in double-wall, thermoregulated cultivation cylinders (internal diameter 35 mm). Here, the culture was maintained at Chl concentration of 6 to 8 μg mL^{-1} by regularly diluting with approximately 10 mL of BG11 medium every 150 min. The culture was bubbled with air containing 2% (v/v) CO_2 and illuminated with white light at 40 μmol photons m^{-2} s^{-1} at 29°C. For the large-scale cultivation used for isolation of PSII complexes, cultures were grown in 10-L flasks (culture volume 6–8 L), stirred by a magnetic stirrer, and bubbled with air.

Measurements of autotrophic growth rates were performed in microtitration plates (culture volume 0.25 mL) under intensive shaking and illumination at 25 μmol photons m^{-2} s^{-1}. Optical densities at 750 nm were measured every 6 h using microplate reader (Tecan Sunrise). Values plotted against time were used for calculation of the doubling time.

Fluorometric and Polarographic Methods

The maximum photochemical efficiency of PSII in the dark-adapted state parameter and kinetics of Chl variable fluorescence decay were measured in dark-adapted cultures (2.5 μg Chl mL^{-1}) using a modulation PAM101 fluorometer (Walz) with an ED-101US cuvette and the Dual-Modulation Kinetic fluorometer (Photon Systems Instruments; Tichý et al., 2003). The light-saturated steady-state rate of oxygen evolution in cell suspensions was measured polarographically in BG11 medium containing 10 mM HEPES/NaOH, pH 7.0, using 0.5 mM p-benzoquinone and 1 mM potassium ferricyanide as artificial electron acceptors.

Preparation of Membranes and Their Protein Analysis

Cyanobacterial membranes were prepared by breaking the cells using glass beads (Komenda and Barber, 1995) with the following modifications: cells were washed, broken, and finally resuspended in 25 mM MES/NaOH, pH 6.5, containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 25% glycerol. The large-scale isolation for chromatographic purification of PSII complexes was performed by a similar procedure, only the cells were resuspended in 20 mL of thylakoid buffer (25 mM MES/NaOH, pH 6.5, 100 mM NaCl) containing the protease inhibitor cocktail (Roche), the same volume of glass beads was added, and the cells were broken eight times for 15 s in the smallest container of beadbeater (Biospec Products) with a 5-min interruption for cooling on ice. Glass beads were subsequently removed by filtering and thylakoids were obtained by differential centrifugation.

For analysis of protein complexes, isolated membranes were solubilized with DM (DM/Chl = 40; w/w) and analyzed by BN electrophoresis at 4°C in 5% to 14% polyacrylamide gel according to Schägger and von Jagow (1991). Samples with the same Chl content (6 μg for gel staining and 1 μg for western blot) were loaded onto the gel.

Protein composition of complexes was assessed by electrophoresis in a denaturing 12% to 20% linear gradient polyacrylamide gel containing 7 M urea (Komenda et al., 2002). The whole lanes from the native gel were excised, incubated for 30 min in 25 mM Tris/HCl, pH 7.5, containing 1% SDS (w/v), and placed on the top of the denaturing gel; two lanes were analyzed in a single denaturing gel. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with specific primary antibodies and then with secondary antibody-horseradish peroxidase conjugate (Sigma). The primary antibodies used in the study were raised in rabbits against: (1) residues 58 to 86 of the spinach (*Spinacia oleracea*) D1 polypeptide; (2) the last 12 residues of the D2 polypeptide from *Synechocystis* 6803; (3) residues 380 to 394 of barley (*Hordeum vulgare*) CP47; (4) the whole isolated CP43 from *Synechocystis* 6803; and (5) the last 14 residues of the PsbI protein from *Synechocystis* 6803. For autoradiography, the gel or the membrane with labeled proteins was exposed to x-ray film at laboratory temperature for 2 to 3 d or to a PhosphorImager plate (GE Healthcare) overnight.

Samples used for direct comparison and quantification of stained or labeled proteins were loaded with the same Chl content and were run on a single gel. Bands of α- and β-subunits of ATP synthase were used as an internal standard to which intensity of bands was related. Quantification of bands was done using ImageQuant 5.2 software (GE Healthcare).

Radioactive Labeling of the Cells

For the radioactive labeling, cells containing 75 μg of Chl were resuspended in 250 μL of BG11 in a microcentrifuge tube, shaken at 60 μmol photons m^{-2} s^{-1} for 30 min, and then a mixture of [^{35}S]Met and [^{35}S]Cys (Trans-label, MP Biochemicals) was added (final specific activity 400 μCi mL^{-1}). The suspension was exposed to light at irradiance and temperature indicated and afterward the cells were frozen in liquid nitrogen and used for isolation of thylakoids.

Coimmunoprecipitation of PsbI and D1

Coimmunoprecipitation was performed using antibodies against the PsbI and the D1 protein as described previously (Komenda et al., 2005). Briefly, thylakoids isolated from radioactively labeled cells of the ΔCYT and ΔCYT/ΔPsbI strain were solubilized with DM and after overnight incubation with the specific antibodies, the immunoglobulins bound to Protein A-Sepharose 4B (Sigma) were released by the Tris buffer containing 2% SDS and 2% dithiothreitol at 50°C. The eluate was analyzed by one-dimensional SDS-PAGE, transferred to PVDF membrane, and used for autoradiography and immunodetection.

Isolation of PSII Complexes

The D1-PsbI-His complex was isolated from thylakoids of PsbI-His/ΔPsbI/ΔCYT using affinity chromatography on immobilized Ni^{2+} ions. Thylakoid membranes were solubilized with DM at a final concentration of 2% (w/v) in thylakoid buffer (25 mM MES/NaOH, pH 6.5, 100 mM NaCl) for 30 min in the dark and on ice. The Chl concentration was 1 mg mL^{-1}. The unsolubilized material was removed by centrifugation for 20 min at 60,000*g* at 4°C and the supernatant was loaded to a chromatography column with Fractogel EMD Chelate (Merck) charged with Ni^{2+} and equilibrated with thylakoid buffer containing 0.04% DM (w/v; Bumba et al., 2005). The column was subsequently washed with the same buffer until the eluate remained colorless and the PSII was eluted using the isolation buffer with an added 20 mM imidazole and 0.04% DM.

Chl Content

For the measurement of Chl concentration, sedimented cells or membranes were extracted with 100% methanol and Chl content in the extract was calculated from the absorbance at 666 and 720 nm (Wellburn and Lichtenthaler, 1984).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Level of the unassembled PsbI protein is controlled by the protease FtsH (slr0228).

Supplemental Figure S2. PsbI does not affect assembly of the PSII RC47 complex in the CP43-less strain.

Supplemental Figure S3. Effect of PsbI absence on levels of the D1 protein in the wild type, *psbB* (ΔCP47), and *psbEFLJ* (ΔCYT) deletion mutants.

Supplemental Table S1. Characteristics of the wild-type strain and the mutant ΔPsbI.

Supplemental Table S2. Distribution of the stained and labeled D1 protein among PSII complexes after pulse and pulse-chase labeling of wild type and the ΔPsbI mutant.

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The Psb28 protein is preferentially associated with the Photosystem II subcomplex lacking CP43 and participates in synthesis of chlorophyll-binding proteins in the cyanobacterium *Synechocystis* sp. PCC 6803

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ABSTRACT

The role of the putative Photosystem II protein component Psb28 in structure and function of Photosystem II has been studied in the cyanobacterium *Synechocystis* sp. PCC 6803. Two-dimensional protein analysis showed that this membrane-associated protein was present mostly in the fraction of unassembled proteins but its small portion was detected in the monomeric PSII core complex and especially in the complex lacking CP43 (RC47). The association of Psb28 with RC47 was confirmed by protein analysis of RC47 isolated from the CP43-less strain and by preferential isolation of RC47 from the strain containing a His-tagged derivative of Psb28 using a nickel affinity chromatography. A *psb28* null mutant exhibited slower autotrophic growth than wild-type but characterization of its photosynthetic apparatus showed that the absence of Psb28 did not affect the functional properties of PSII complex. Although the cells of the mutant were not high light sensitive, they exhibited slightly accelerated turnover of the D1 protein and the faster PSII repair. Radioactive labeling of proteins indicated limitation in the synthesis of CP47 and Photosystem I (PSI) in the mutant. Relationship between Psb28 and CP47 was further confirmed by a substantial decrease in the level of unassembled CP47 in the *psb28/psbEFLJ* double mutant which is unable to synthesize D2 and hence cannot assemble PSII reaction center complex. Moreover, all *psb28* deletion mutants released large quantities of chlorophyll breakdown products into the medium. Overall our results suggest that Psb28 participates in the biogenesis of chlorophyll-proteins, most probably in the process of chlorophyll binding to corresponding apoproteins. Its binding to RC47 tempts to speculation that Psb28 participates in the turnover of the D1 protein. It might support re-binding of chlorophyll molecules released from the degraded D1 to the newly synthesized D1. As a consequence, the new chlorophyll molecules are not bound to D1 and instead they are utilized for synthesis of other chlorophyll-proteins, namely CP47 and PSI.

Introduction

The Photosystem II (PSII) complex is the multi-subunit membrane protein complex catalyzing oxidation of water and reduction of plastoquinone in the thylakoid membranes of higher plants, algae and cyanobacteria. In the heard of the complex there are two similar membrane spanning proteins D1 and D2 that bind the cofactors involved in primary charge separation (Nanba and Satoh, 1987) and subsequent electron transfer within PSII subunits (for recent review see Barber, 2006). Peripherally to the D1-D2 heterodimer there are two inner chlorophyll (Chl)-binding antenna proteins CP47 and CP43 that deliver energy to the reaction center (RC) for driving electron transfer. In addition, CP43 also provides important ligands to the Mn_4Ca cluster, the site of water oxidation. These four large proteins are surrounded by a number of smaller membrane polypeptides (for recent review see Shi and Schröder, 2004). One of them, the so called PsbW was originally detected in the isolated RC complex from spinach (Ikeuchi et al., 1989). The mature protein with a predicted a transmembrane α-helix in the central hydrophobic region seems to have (unlike most of PSII membrane proteins) the N-terminus oriented into the lumen in close vicinity to the extrinsic, nuclear-encoded 33 kDa PsbO protein (Irrgang et al., 1995; Lorković et al., 1995). Cross-linking experiments indicated a close association of PsbW with D1, D2 and α subunit of the cytochrome b-559 (cyt b-559) in the isolated RC complex (Irrgang et al., 1995; Lorković et al., 1995). At variance with these results, Rokka et al. (2005) located PsbW predominantly in PSII-LHCII supercomplexes and only minor amounts were found in PSII core dimers and monomers. In transgenic plants of *Arabidopsis thaliana* lacking the PsbW protein the stability of the dimeric PSII was diminished and the PSII-LHCII supercomplexes could not be detected (Shi et al., 2000). It has been suggested that PsbW functions as a linker for LHCII binding to the PSII complex. As LHCII is absent in cyanobacteria, it was intelligible that the PsbW was not detected in these oxygenic autotrophs. Nevertheless, N-terminal sequencing and mass spectrometric analyses of protein subunits in the purified His-tagged PSII from *Synechocystiis* sp. PCC 6803 (Synechocystis 6803) revealed the presence of a unknown protein with the 16% sequence identity to the *Arabidopsis* plant PsbW (Kashino et al., 2002). This protein was designated as Psb28 (also Psb13 or ycf79). Its amino acid sequence suggests that it is a rather hydrophilic protein without transmembrane helix (http://bacteria.kazusa.or.jp/ cyanobase/Synechocystis/) and is larger than PsbW (about 13 kDa). In the recent crystal structures of cyanobacterial PSII (Ferreira et al., 2004; Loll et al., 2005) this protein was not identified and remains open if the

protein is a true PSII subunit, a transiently associated assembly factor or just an impurity of the preparation. The relatively low content of this protein in the isolated preparation suggested that the two latter possibilities are more probable. Very recently, the protein has been detected as a component of PSII complexes in *Synechocystis* 6803 depleted of phosphatidylglycerol (Sonoike et al., 2007). It has been proposed that the protein may play a regulatory role during PSII assembly of Photosystem II. A gene encoding a soluble protein with the C-terminal domain having 44% identity to Psb28 of *Synechocystis* 6803 has also been found in the genome of *Arabidopsis* and the protein was designated PsbW-like.

Here we present a detailed analysis of the role of Psb28 in the structure and function of the cyanobacterial PSII. The results showed that Psb28 is not a component of the fully assembled dimeric PSII core complex but it is preferentially bound to the PSII core complex lacking antenna CP43 where seems to play a role in recycling chlorophyll during the selective turnover of D1. We hypothesize that in the absence of Psb28 an extensive consumption of new chlorophyll for the resynthesis of D1 leads to deficiency in synthesis of other chlorophyll-binding proteins like CP47 and PSI.

RESULTS

The membrane-associated fraction of Psb28 is preferentially bound to the PSII subcomplex lacking CP43

Psb28 was identified as a minor component of PSII complexes purified using nickel affinity chromatography from the strain expressing His-tagged CP47 (Kashino et al., 2002). To provide more rigorous information of the localization of Psb28, we first screened for its presence in the membrane-associated and soluble protein fractions of the whole cell extract using specific antibodies raised against the last 15 amino acid residues of the protein (Fig. 1A). The vast majority of the protein was found in the membrane-bound fraction but more than 90% of its content was removed by the treatment using 1M $CaCl_2$ and 1M sodium carbonate or 0.1M sodium hydroxide washed out the protein completely (Fig. 1B). 90% of the protein was also removed by the treatment with trypsin which in this type of membrane preparation acts only on the stromal side cleaving the D1 protein to specific fragments (Komenda et al., 2002). Thus, Psb28 is the protein peripherally associated with the stromal side of the membranes. To identify the membrane binding site of Psb28 we analyzed

membrane protein complexes of wild-type (WT) using 2D separation of membrane proteins consisting of blue native PAGE in one direction and denaturing PAGE in the second direction (2D BN/SDS-PAGE). Majority (80-90 %) of Psb28 was detected as a well defined spot at the end of the BN gel in the low-molecular-weight region indicating that it is not assembled in any large membrane complex (Fig. 2A, blot). However, a small portion of the protein was found in the position corresponding to the migration of a PSII core subcomplex lacking CP43 (termed RC47) while the dimeric and monomeric core complexes RCC(2) and RCC(1) were seemingly free of this subunit. As WT contains only a small amount of RC47, Psb28 bound to this complex was usually hardly detectable. Therefore, we also analyzed PSII complexes in the phycobiliprotein-free strain PAL which exhibits much higher PSII/PSI ratio (Ajlani and Vernotte, 1998) and also in the site-directed mutant D1-Asn359His containing a rather high amount of RC47 (Kuviková et al., 2005). 2D analysis confirmed the presence of Psb28 in RC47 of PAL, the protein was apparent even in the stained gel (Fig. S1A, arrow) and its identity was verified by de novo sequencing of the band using Q-TOF mass spectrometry. However, the protein staining showed that a small amount of the protein might be associated also with the monomeric PSII core complex RCC(1). In the case of the site-directed mutant D1-Asn359His blotting confirmed the presence of the protein in RC47 and again the small amount of Psb28 was also found in the monomeric core complex RCC(1) (Fig. S1B).

To further confirm the preferential association of Psb28 with RC47, we isolated the RC47 complex from the mutant lacking CP43. A combination of copper affinity chromatography and ionex chromatography on Q Sepharose (Komenda et al., 2002) yielded well purified complex that contained nearly stoichiometric amount of Psb28 as indicated by the similar staining intensity of Psb28 band in comparison with the band of the α subunit of cyt b-559 (Fig. 2A).

The third line of evidence for binding of Psb28 to RC47 was obtained using a strain in which the original *psb28* gene was replaced by a His-tagged copy. Thylakoids from the Psb28-His/ΔPsb28 strain were solubilized with dodecyl-maltoside (DM) and the extract was loaded on the Ni-affinity chromatography column. Bound proteins were analyzed by 2D BN/SDS-PAGE and immunoblotting using antibodies against Psb28, D1, CP47 and CP43. The majority of Psb28-His was present as free protein but its substantial portion was found in RC47 (Fig. 2B). The isolated preparation also contained small amount of RCC(1) (Fig. 2A), nevertheless its content in the preparation was always below 20% of the RC47 amount confirming the preferential interaction between Psb28-His and RC47.

Absence of Psb28 affects synthesis of chlorophyll proteins CP47 and PsaA/PsaB

To characterize the function of Psb28 we constructed a *psb28*-deletion mutant (ΔPsb28) in which the *psb28* gene was replaced by a zeocine-resistance cassette. The resulting mutant exhibited a slower photoautotrophic growth at three different growth irradiances but its PSII photochemical activity, measured as the variable fluorescence yield or the light-saturated rate of oxygen evolution, were similar to WT (Tab. 1). Also the rates of Q_A reoxidation measured in the presence and absence of PSII inhibitor diuron were identical in both strains. This result corresponded well with the observed absence of the protein in the dimeric PSII core complexes and showed that the protein is not important for functioning of the fully assembled photochemically active PSII complexes.

To characterize PSII assembly in WT and ΔPsb28, we pulse-labeled the cells with [^{35}S]Met/Cys and analyzed their membrane protein complexes and incorporation of radiolabel into the protein subunits by 2D BN/SDS-PAGE. In both strains more than 90% the D1 subunit was present in the dimeric and monomeric core complexes, RCC(2) and RCC(1) by staining with Coomassie-blue (Fig. 3A). In WT at least 10% of D1 was present in the RC47 while in ΔPsb28 this complex was less abundant. CP47 was distributed among complexes of WT similarly to D1 and only about 5% of overall level was found in the fraction of unassembled proteins (Fig. 3A, arrow 1) but no free CP47 was found in ΔPsb28. The overall amount of the stained PSII proteins in the gel loaded on the same chlorophyll basis was 20 to 40% higher in the mutant than in WT and this was confirmed for all four large PSII proteins D1, D2, CP43 and CP47 by a semiquantitative western blot (Fig. 3B).

Autoradiograms obtained from the same 2D gels were used to assess the synthesis of various membrane proteins and their assembly into complexes. In WT the D1 protein was preferentially labeled in both RCC(2) and RCC(1). There was also apparent labeling of D1 in RC47 but not in the RC complexes containing D1, D2, cyt b-559 and PsbI (Fig. 3A; autorad; Dobáková et al., 2007). In contrast, in membranes of ΔPsb28 about 20 % of labeled D1 was detected in RCa complex and 30 % in the fraction of unassembled proteins (Fig. 3A, autorad, arrow 2). Previous analyses of various *Synechocystis* 6803 mutants (Komenda et al., 2004; Komenda et al., 2005 and Dobáková et al., 2007) showed that the presence of RC complexes and unassembled D1 protein indicates that the PSII assembly is limited by availability of CP47. In agreement with this interpretation the labeled unassembled CP47 was present in WT but absent in ΔPsb28. Surprisingly, the absence of Psb28 also negatively affected the synthesis of other large chlorophyll-binding subunits, the PsaA/PsaB heterodimer of

Photosystem I. The radioactive labeling of this heterodimer in WT several times exceeded that in ΔPsb28 (Fig. 3A; autorad; arrow 3). Overall, the results of radioactive labeling suggested that Psb28 is needed for the efficient synthesis of chlorophyll proteins CP47 and PSI.

The results of the protein analyses suggested higher ratio PSII/PSI in the mutant and this was also supported by the 77K chlorophyll fluorescence emission spectra of cells from both strains (Fig. 4A). The fluorescence maxima at 685 and 693 nm in the spectra normalized to the main peak of PSI fluorescence at 725 nm were apparently higher in the mutant cells when compared with the WT cells.

An importance of Psb28 for the biogenesis of chlorophyll-proteins was further supported by absorption spectroscopy. Absorption spectra of WT and ΔPsb28 normalized to cell number showed lower chlorophyll level in the mutant cells (compare 680 nm maxima in the WT and mutant spectra) and another difference was found in the region from 350 to 430 nm in which the mutant exhibited much higher absorption. This absorption was caused by the presence of compounds excreted by the mutant cells into the growth medium. Indeed, there was only a small difference between the spectra of cells measured after their washing and resuspending in the fresh medium (Fig. 4B). Similar compounds were also produced in a strain lacking genes encoding chlorophyll-binding proteins CP47, PsaA and PsaB suggesting that they might represent chlorophyll metabolites accumulating under conditions when the pigment cannot bind to apoproteins. The absorption spectra of supernatant of ΔPsb28 after sedimenting the cells exhibited major peaks at 320, 360 and 410 nm. While in fresh cultures with young cells the first peak dominated, in the later stage of growth the peak at 410 nm gradually increased. To characterize an origin of the absorbing substances we analyzed the medium after sedimenting the cells by LC-MS. We separated two main fractions, the first one reddish with absorption maxima 320 and 360 nm and prevailing ion species m/z = 256 followed by the yellow fraction having the absorption maximum at 410 nm with shoulder at 365 nm and the main ion species 390. The masses and fragmentation pattern was in agreement with the identity of the separated molecules as derivatives of the connected rings III, IV and V of the chlorin cycle of chlorophyll differing in the oxidation level of its side chains. The data suggested that in the absence of Psb28 large quantities of chlorophyll are degraded and the degradation products secreted outside the cells. Taking together with inhibition of synthesis of PSI and CP47 in the absence of Psb28 it appears that Psb28

promotes utilization of chlorophyll for synthesis of PSI and CP47. In contrast, synthesis of the chlorophyll-proteins CP43 and D2 did not seem to be affected.

We also constructed three double mutants in which the deletion of the *psb28* gene was performed in strains lacking the genes encoding PSII proteins: *psbB, psbC* and the *psbEFLJ* operon. In all these strains excretion of chlorophyll breakdown products was observed and the strains exhibited limited synthesis of the PsaA/B heterodimer. In the strain assembling only the RC47 complex due to the deletion of the *psbC* gene encoding CP43, an additional inactivation of the *psb28* gene led to the 50% decrease in the level of RC47 and the level of unassembled CP47 became negligible. In the *psbEFLJ/psb28* double mutant which is unable to synthesize cyt b-559 and the D2 protein (Komenda et al., 2004). The limitation in the synthesis of CP47 led to a substantial decrease in the steady state level of the unassembled CP47 while the accumulation of the unassembled D1 was increased.

Psb28 does not affect high light sensitivity but its absence accelerates turnover of the D1 protein

Given that Psb28 is a preferential component of RC47 in which the selective replacement of D1 most probably takes place (Komenda et al., 2004 and 2006), we were interested to learn whether the absence of Psb28 affects D1 turnover. Pulse-chase experiments (Fig. 5) showed that the turnover of D1 was slightly accelerated in the absence of Psb28. Pulse-chase labeling revealed a half-life of D1 in WT of about 2.5 h, while in the mutant ΔPsb28 it was approximately 2 h. To find out if the resistance of PSII photochemistry to photodamage was also similar in both strains, we evaluated the time course of light-induced inhibition of oxygen evolution in WT and mutant cultures subjected to white light of 500 µmol photons $m^{-2}.s^{-1}$ either in the absence or presence of lincomycin. Cultures of WT and ΔPsb28 did not exhibit any differences in the decline of oxygen evolution despite the absence or presence of lincomycin (Fig. 6A). Recovery from photoinhibition under low light conditions was even faster in ΔPsb28 than in WT as evidenced by assessment of oxygen evolution in photoinhibited cells of WT and ΔPsb28 during subsequent incubation at 50 µmol photons $m^{-2}.s^{-1}$ (Fig. 6B). The results showed that the oxygen-evolving PSII complexes of the mutant are equally sensitive to light-induced inactivation as complexes of WT while the repair of PSII seems to be slightly more efficient and this corresponds with the accelerated turnover of D1 observed in the strain.

We also checked if the protein is needed to protect PSII proteins from oxidative damage under extreme light conditions (2000 µmol photons $m^{-2}.s^{-1}$). While in WT cells these conditions did not lead to the significant changes in their absorption spectra and the content of PSII proteins, the exposure of the ΔPsb28 to these light conditions resulted in a significant decline in the level of D1 although remaining D1 did not exhibit symptoms of oxidation like smearing or shift to the higher molecular weight (Komenda et al., 2002). Similar results were obtained with the strain lacking one of the SCP proteins, ScpB.

Discussion

Role of Psb28 in the structure and function of Photosystem II

The Psb28 protein has been previously detected as a minor component of the PSII preparation purified using Ni-affinity chromatography from *Synechocystis* 6803 strain expressing His-tagged CP47 (Kashino et al., 2002). However, this method is able to purify all PSII complexes containing CP47-His including RCC(2), RCC(1), RC47 and unassembled CP47 and therefore it was not clear to what PSII complex Psb28 actually binds. Very recently, Sakurai et al. (2007) have also detected the protein in the isolated monomeric PSII complex. However, this complex was obtained by glycerol density gradient centrifugation which has insufficient separation ability to resolve RCC(1) and RC47, therefore the putative monomeric PSII most probably contained both complexes. Here we show that Psb28 binds preferentially to RC47 and only the small amount of Psb28 was occasionally found also in the monomeric RCC(1) complex. However, we never found the protein in the dimeric complex RCC(2) which is considered as the native, functionally fully competent form of PSII (Barber, 2006). In accordance with this the functional properties of PSII in the *psb28* deletion mutant do not seem to be affected by the absence of the protein. The presence of Psb28 in RCC(1) was typical for strains containing a large amount of RC47 indicating that the protein most probably remains attached for a limited time after binding of CP43 to RC47.

Role of the Psb28 protein in the chlorophyll metabolism and synthesis of chlorophyll binding proteins

The characteristic feature of all constructed *psb28* deletion mutants is the increased production of the chlorophyll degradation products indicating defect in coordination between the synthesis of chlorophyll and synthesis of corresponding apoproteins. Thus, Psb28 seems to be important for this coordination. Moreover, its preferential occurrence of in the RC47 complex, in which the selective replacement of D1 most probably occurs (Komenda et al., 2004; Komenda et al., 2006), indicates that coordination during the D1 replacement miht be affected by the absence of Psb28. Recent results showing association of so called small Cab-like proteins (SCPs') with PSII (Promnares et al., 2006; Yao et al., 2007) and their presumable role in the temporary binding of chlorophyll during the D1 replacement (Vavilin et al., 2007) indicate that chlorophyll molecules released from the degraded D1 are largely re-used for binding to the new D1 molecule. PSII is potentially more intensive consumer of chlorophyll than PSI due to the PSII repair cycle based on the D1 turnover and in the absence of SCPs' an intensive degradation of chlorophyll during the D1 turnover and release of its degradation products similar to those found in the Psb28-less strains is expected. Indeed, when we compared the absorption spectra of the cell cultures of WT, the *scpBCDE*-less quadruple deletion mutant and ΔPsb28 exposed to high irradiance, a similar increase in the absorption around 410 and 360 nm was observed in both mutants but not in WT. Moreover, in the mutants symptoms of oxidation damage to D1 were observed which could be ascribed to the photodynamic action of released chlorophyll. It is tempting to speculate that Psb28 acts as a factor supporting recycling chlorophyll during the PSII repair. If not present, the chlorophyll released from the degraded D1 cannot be reused and the new D1 protein must use the newly synthesized chlorophyll molecules. A consequence of this defect is an insufficient supply of chlorophyll for synthesis of the chlorophyll-binding proteins like CP47 and PSI which is typical for all constructed *psb28* deletion mutants.

Material and Methods

Construction and cultivation of cyanobacterial strains

The strains used in the study were derived from the glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (Williams, 1988) referred to as wild-type. The following, previously described strains were used in the study: (i) the CP43-less strain ΔCP43 with the *psbC* gene inactivated by kanamycin resistance cassette (Vermaas et al., 1988); (ii) the CP47-less strain ΔCP47 with the *psbB* gene inactivated by spectinomycin resistance cassette (Eaton-

Rye and Vermaas, 1991); and (iii) the ΔCYT strain with the *psbEFLJ* operon replaced by kanamycin resistance cassette (Pakrasi et al., 1988).

The ΔPsb28 strain was prepared by replacement of most of the *psb28* gene (nucleotides 18-298) by a zeocine resistance cassette using megaprimer PCR method (Burke and Barik, 2003). We have adapted this method to generate linear deletion constructs in vitro containing upstream and downstream regions of the *psb28* gene with the zeocine resistance cassette in the middle. In the first step, upstream and downstream regions of *psb28* were separately amplified using long fusion primers recognizing in one direction the *psb28* gene and in the other direction the zeocine cassette: (i) 5'-ACATTAATTGCGTTGCG CTCACTGCAAATTGAATTTCAGCCATG-3' and (ii) 5'-CAACTTAATCGCCTT GCAGCACATCGGCGCAGAAAATGGCTTAGG-3'(the *psb28* part is underlined). These fusion primers were used in pairs with *psb28* forward and reverse primers: (i) 5'-TGTTCTACCTGCTCGATCGC-3' and (ii) 5'-GAGTTTCGCTACTATCAGCG-3'. In the second step the zeocine resistance cassette (*Streptoalloteichus hindustanus*, Invitrogen, Paisley, UK) was amplified using PCR products from the first step as primers. Finally, the complete deletion construct was amplified using *psb28* forward and reverse primers and used for transformation of *Synechocystis* 6803 cells. Transformants were selected and segregated on zeocine-containing agar plates; their full segregation was confirmed by PCR.

Multiple *psb28* deletion strains were obtained by transformation of single mutants lacking *psbC, psbB, psbEFLJ* and *psbA* genes using chromosomal DNA from ΔPsb28 and their selection for the additional resistance to zeocine.

The Psb28-His strain expressing Psb28 tagged with the 6xHis tag at its N-terminus under control of the *psbA2* promoter (Psb28-His/ΔPsb28) was constructed using the *pSBA* plasmid (Lagarde et al., 2000) in a procedure analogous to that described in (Tichý et al., 2003). The resulting strain synthesizing both WT and His-tagged forms of Psb28 was transformed with chromosomal DNA from the *psb28* deletion mutant and selected for the resistance to zeocine. The complete deletion of the *psb28* WT copies in the Psb28-His strain was confirmed by PCR. The strain Psb28-His/ΔPsb28/ΔCYT was obtained by transformation of Psb28-His/ΔPsb28 using chromosomal DNA from the *psbEFLJ* deletion strain and selection for the additional resistance to kanamycin.

Liquid cultures were grown in 100 – 200 mL BG11 using 500 mL conical flasks, aerated using an orbital shaker, irradiated with 30 µmol photons m^{-2} s^{-1} of white light at 29 °C and were used when they reached a Chl concentration of about 5 µg.mL^{-1}. Solid medium

contained in addition 10 mM Tes/NaOH, pH 8.2, 1.5 % agar and 0.3 % sodium thiosulphate (Pakrasi et al., 1988). Media for cultivation of non-autotrophic strains contained in addition 5 mM glucose.

Photoinhibition experiments were performed with cells cultivated in double-wall, thermoregulated cultivation cylinders (internal diameter 35 mm). Here, the culture was maintained at Chl concentration of 6-8 µg.mL^{-1} by regularly diluting with approx. 10 mL of BG-11 medium every 150 min. The culture was bubbled with air containing 2 % (v/v) CO_2 and illuminated with white light at 40 µmol photons m^{-2}·s^{-1} at 29 °C. For the large-scale cultivation used for isolation of PSII complexes, cultures were grown in 10 l flasks (culture volume 6-8 l), stirred by a magnetic stirrer and bubbled with air.

Measurements of autotrophic growth rates were performed in microtitration plates (culture volume 0.25 mL) under intensive shaking and illumination at 25 µmol photons m^{-2}.s^{-1}. Optical densities at 750 nm were measured every 6 hours using microplate reader (Tecan Sunrise, Vienna, Austria). Values plotted against time were used for calculation of the doubling time.

Fluorometric and polarographic methods

The F_V/F_M parameter and kinetics of Chl variable fluorescence decay were measured in dark-adapted cultures (2.5 µg Chl.mL^{-1}) using a modulation PAM101 fluorometer (Walz, Effeltrich, Germany) with an ED-101US cuvette and the Dual-Modulation Kinetic Fluorometer (Photon Systems Instruments, Brno, Czech Republic) (Tichý et al., 2003). The light-saturated steady-state rate of oxygen evolution in cell suspensions was measured polarographically in BG-11 medium containing 10 mM Hepes/NaoH, pH 7.0 using 0.5 mM p-benzoquinone and 1 mM potassium ferricyanide as artificial electron acceptors.

Preparation of membranes and their protein analysis

Cyanobacterial membranes were prepared by breaking the cells using glass beads (Komenda and Barber, 1995) with the following modifications: cells were washed, broken and finally resuspended in 25 mM Mes/NaOH, pH 6.5 containing 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 25 % glycerol. The large-scale isolation for chromatographic purification of PSII complexes was performed by a similar procedure only the cell were resuspended in 20 mL of thylakoid buffer (25 mM Mes, pH 6.5, 100mM NaCl) containing the protease inhibitor cocktail (Roche, Mannheim, Germany), the same volume of glass beads was added and the cells were broken 8 times for 15 s in the smallest container of beadbeater (Biospec Products,

Bartlesville, USA) with 5 min interruption for cooling on ice. Glass beads were subsequently removed by filtering and thylakoids were obtained by differential centrifugation.

For analysis of protein complexes isolated membranes were solubilized with DM (DM/Chl = 40, w/w) and analyzed by blue-native electrophoresis at 4ºC in 5-14 % polyacrylamide gel according to Schägger and von Jagow (1991). Samples with the same Chl content (6 µg for gel staining and 1 µg for Western blot) were loaded onto the gel.

Protein composition of complexes was assessed by electrophoresis in a denaturing 12-20 % linear gradient polyacrylamide gel containing 7 M urea (Komenda et al., 2002). The whole lanes from the native gel were excised, incubated for 30 min in 25 mM Tris/HCl, pH 7.5 containing 1 % SDS (w/v) and placed on the top of the denaturing gel; two lanes were analyzed in a single denaturing gel. Proteins separated in the gel were either stained by Coomassie Blue or transferred onto PVDF membrane. Membranes were incubated with specific primary antibodies and then with secondary antibody-horseradish peroxidase conjugate (Sigma, St. Louis, USA). The primary antibodies used in the study were raised in rabbits against: (i) residues 58-86 of the spinach D1 polypeptide, (ii) the last 12 residues of the D2 polypeptide from *S.* 6803, (iii) residues 380-394 of barley CP47; (iv) the whole isolated CP43 from *Synechocystis* 6803; and (v) the last 15 residues of the Psb28 protein from *Synechocystis* 6803. For autoradiography, the gel or the membrane with labeled proteins was exposed to X-ray film at laboratory temperature for 2-3 d or to Phosphorimager plate (GE Healthcare, Vienna, Austria) overnight.

Samples used for direct comparison and quantification of stained or labeled proteins were loaded with the same Chl content and were run on a single gel. Quantification of bands was done using ImageQuant 5.2 software (GE Healthcare, Vienna, Austria).

Radioactive labeling of the cells

For the radioactive labeling, cells containing 75 µg of Chl were resuspended in 250 µl of BG 11 in an Eppendorf tube, shaken at 60 µmol photons $m^{-2}.s^{-1}$ for 30 min and then a mixture of $[^{35}S]$Met/Cys (Trans-label, MP Biochemicals, Irvine, USA) was added (final specific activity 400 $\mu Ci.mL^{-1}$). The suspension was exposed to light at irradiance and temperature indicated and afterwards the cells were frozen in liquid nitrogen and used for isolation of thylakoids.

Isolation of PSII complexes

The Psb28-His containing PSII complexes were isolated from thylakoids of Psb28-His/ΔPsb28 using affinity chromatography on immobilized Ni^{2+} ions. Thylakoid membranes were solubilized with DM - final concentration 2% (w/v) in thylakoid buffer (25mM Mes, pH 6.5, 100mM NaCl) for 30 min in the dark and on ice. The Chl concentration was 1 $mg.mL^{-1}$. The unsolubilized material was removed by centrifugation for 20 min at 60,000 g at 4°C and the supernatant was loaded to a chromatography column with Fractogel EMD Chelate (Merck, Darmstadt, Germany) charged with Ni^{2+} and equilibrated with thylakoid buffer containing 0.04% DM (w/v) (Bumba et al., 2005). The column was subsequently washed with the same buffer until the eluate remained colorless and the PSII complexes were eluted using the isolation buffer with added 20 mM imidazole and 0.04% DM.

The RC47 complex was isolated from the *psbC/slr0228* double deletion mutant (Komenda et al., 2006) by a combination of metal affinity chromatography on Chelating Sepharose Fast Flow (Amersham Biosciences) with immobilized Cu^{2+} ions and ionex chromatography on Q Sepharose (Amersham Biosciences) (Komenda et al., 2002).

Chl Content

For the measurement of Chl concentration, sedimented cells or membranes were extracted with 100% methanol and Chl content in the extract was calculated from the absorbance at 666 and 720 nm (Wellburn and Lichtenthaler, 1984).

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Figure legends

Figure 1: Presence of the Psb28 protein in the membrane-associated and soluble protein fraction. (A) Whole cell extract, membrane-associated and soluble protein fractions were separated by SDS-PAGE proteins in the gel were stained by Coomassie blue (stained gel), blotted onto PVDF membrane and immunodetected by anibodies against the D1 protein and Psb28. **(B)** Thylakoid membranes treated using 1M $CaCl_2$ and 1M sodium carbonate or 0.1M sodium hydroxide and trypsin were separated by SDS-PAGE, blotted onto PVDF membrane and the amount of D1 and Psb28 was quantified by anibodies against the D1 protein and Psb28.

Figure 2: Identification of Psb28 protein in the isolated RC47 complex from the *psbC/slr0228* double mutant (A) and in the PSII complexes from the Psb28-His/ΔPsb28 strain (B). (A): The RC47 complex isolated by a combination of metal affinity and ionex chromatography from the *psbC/slr0228* double mutant was analyzed by 1D SDS-PAGE. The gel was stained and identity of the proteins confirmed by mass spectrometry. **(B)**: PSII complexes isolated by metal affinity chromatography from the Psb28-His/ΔPsb28 strain expressing the His-tagged Psb28 were analyzed by 2D BN/SDS-PAGE and proteins were blotted onto PVDF membrane, stained (blot stain) and probed with antibodies against CP47, D1 and Psb28.

Figure 3: 2D analysis of membrane protein complexes (A) and quantification of photosynthetic membrane proteins (B) of WT and the *psb28* deletion strain ΔPsb28. (A) Cells of WT and ΔPsb28 grown in the absence of glucose were radiolabeled at 500 µmol photons m^{-2} s^{-1} and 30 °C with a mixture of [^{35}S]Met/Cys for 20 min. Designation of complexes: RCC(2) and RCC(1), dimeric and monomeric PSII core complexes, respectively; RC47, PSII core complex lacking CP43; RCa, reaction center complex. U.P. indicates unassembled proteins. Arrows designate important differences between the strains in the accumulation of CP47 and RCa complex and in the labeling of PSI. 6 µg of Chl was loaded for each sample. (B) Membranes of both strains were separated by denaturing SDS-PAGE and electroblotted onto PVDF membrane. Proteins were detected using antibodies specific for CP47, CP43, D2, D1, Psb28 and PsaD proteins of Synechocystis sp. PCC 6803. Correct protein loading was proven by staining of α and β subunits of ATP synthase (AtpAB). 2, 1 and 0.5 µg of chlorophyll were loaded onto the gel.

Figure 4: 77 K fluorescence emission spectra (A) and room-temperature absorption spectra (B) of cells of WT and the *psb28* deletion mutant ΔPsb28

A: Absorption spectra of cells of WT (solid line) and ΔPsb28 (dashed line) cultivated in the absence of glucose. The spectra were obtained using spectrophotometer UV-3000 (Shimadzu, Japan) with cell suspensions of equal OD_{753nm}.

B: 77 K fluorescence spectra of cells of WT (solid line) and ΔPsb28 (dashed line) cultivated in the absence of glucose. The spectra were obtained using Aminco Bowman Series 2 luminescence spectrometer (Spectronic Unicam, USA). The cells were excited at 435 nm, spectra were corrected for the sensitivity of photomultiplier and normalized to the maximum of Photosystem I emission at 725 nm.

Figure 5: Degradation of the PSII proteins in the WT and ΔPsb28 strains under high irradiance monitored by radioactive pulse-chase labeling. Cells of both strains were subjected to 250 µmol photons m^{-2} s^{-1} of white light for 20 min in the presence of [^{35}S]Met/Cys. Then the cells were washed, supplemented with cold Met/Cys and subjected to 500 µmol photons m^{-2} s^{-1} of white light for 6 hours. Thylakoids were isolated, analyzed by SDS-PAGE, the gel was stained (Gel stain) and the radioactive labeling of the proteins was visualized using a PhosphorImager (Autorad.). Quantification of radioactivity in the D1 band was performed by ImageQuant software with samples of each strain equally loaded on Chl basis (2 µg of Chl; see Gel stain) in a single gel. The radioactivity incorporated into the D1 band of each strain during pulse was taken as 100%, numbers show means of three measurements, SD did not exceed 6%.

Figure 6: PSII repair under high and low irradiance in cells of WT and ΔPsb28. (A) Cells of WT (circles) and ΔPsb28 (squares) were illuminated with 500 µmol photons m^{-2} s^{-1} of white light for 180 min in the absence (left panel) or for 120 min in the presence (right panel) of 100 µg mL^{-1} lincomycin and during illumination PSII oxygen-evolving activity was assayed in whole cells. **(B)** Cells of WT (circles) and ΔPsbI (squares) were illuminated at 2000 µmol photons m^{-2} s^{-1} for 20 min (PI), then the cells were transferred to low irradiance of 50 µmol photons m^{-2} s^{-1} and incubated for additional 120 min (REC). During these light regimes PSII oxygen-evolving activity was assayed in whole cells. Values in the plots represent mean of 3 measurements ± SD. Initial values for WT and ΔPsbI were in the range shown in Tab.1.

Figure S1: Identification of Psb28 in the PSII complexes of the phycobilisome-less strain PAL (A) and the site-directed mutant D1-Asn359His (B). Membrane proteins of both strains were separated by 2D BN/SDS-PAGE. PAL proteins in the gel were stained by Coomassie blue, proteins of the D1-Asn359His mutant were blotted onto PVDF and immunodetected by anibodies against the D1 protein and Psb28. The arrow indicates the stained Psb28 protein which was identified by mass spectrometry. 6 µg of Chl was loaded for each sample.

Table 1: Characteristics of the *Synechocystis* sp. PCC 6803 WT and the *psb28* deletion mutant ΔPsb28

Strains	Autotrophic doubling time (h)[a]			Oxygen evolution (µmol O_2. mg $Chl^{-1}.h^{-1}$)[b]	F_V/F_M[c]
	10 µmol	25 µmol	200 µmol		
WT	18.3 ± 0.1	8.3 ± 0.1	8.0 ± 0.1	606 ± 12	0.45 ± 0.02
ΔPsb28	22.8 ± 0.2	11.4 ± 0.1	12.8 ± 0.5	601 ± 14	0.45 ± 0,01

[a]measured in microtitration plates at iiradiances 10, 25 and 200 µmol photons m^{-2} s^{-1} ; means of 10 measurements ± SD; initial $OD_{750\ nm}$ of the cultures was 0.005.

[b]light-saturated rate of oxygen evolution measured in the presence of 1 mM p–benzoquinone and 5 mM potassium ferricyanide; means of 3 measurements ± SD.

[c]values obtained using PAM modulated fluorimeter, F_M was elicited by continuous illumination in the presence of 10^{-5} M diuron ; means of 3 measurements ± SD.

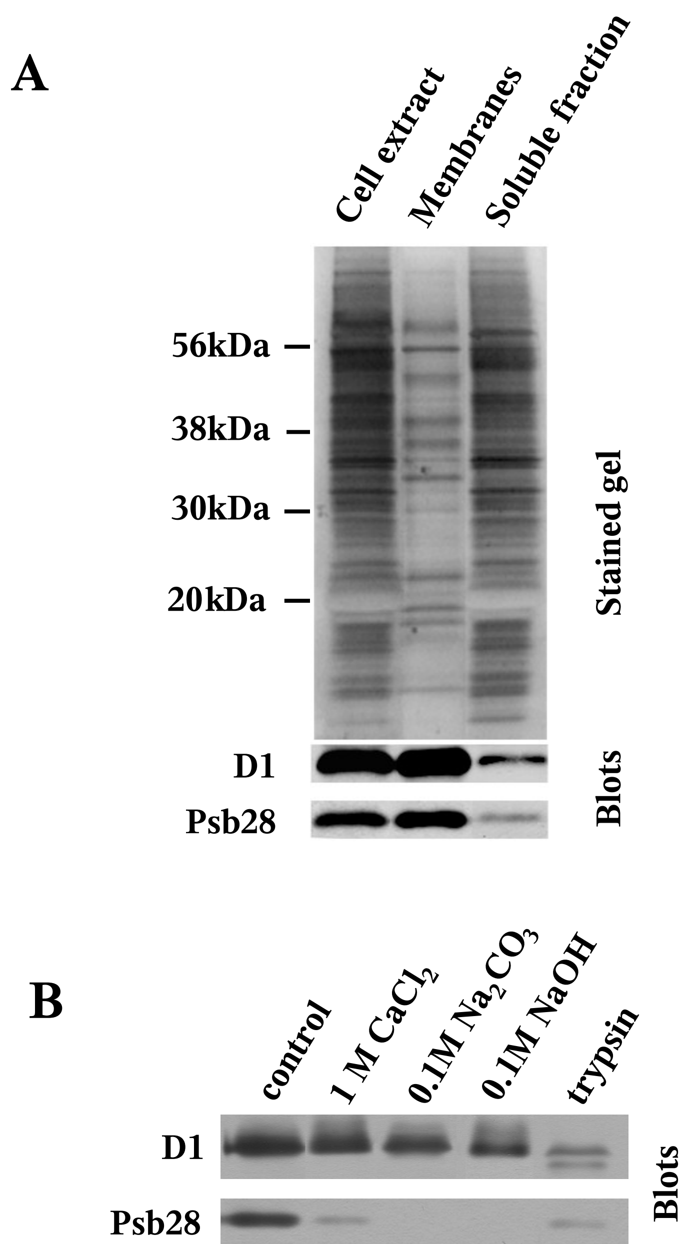

Fig. 1

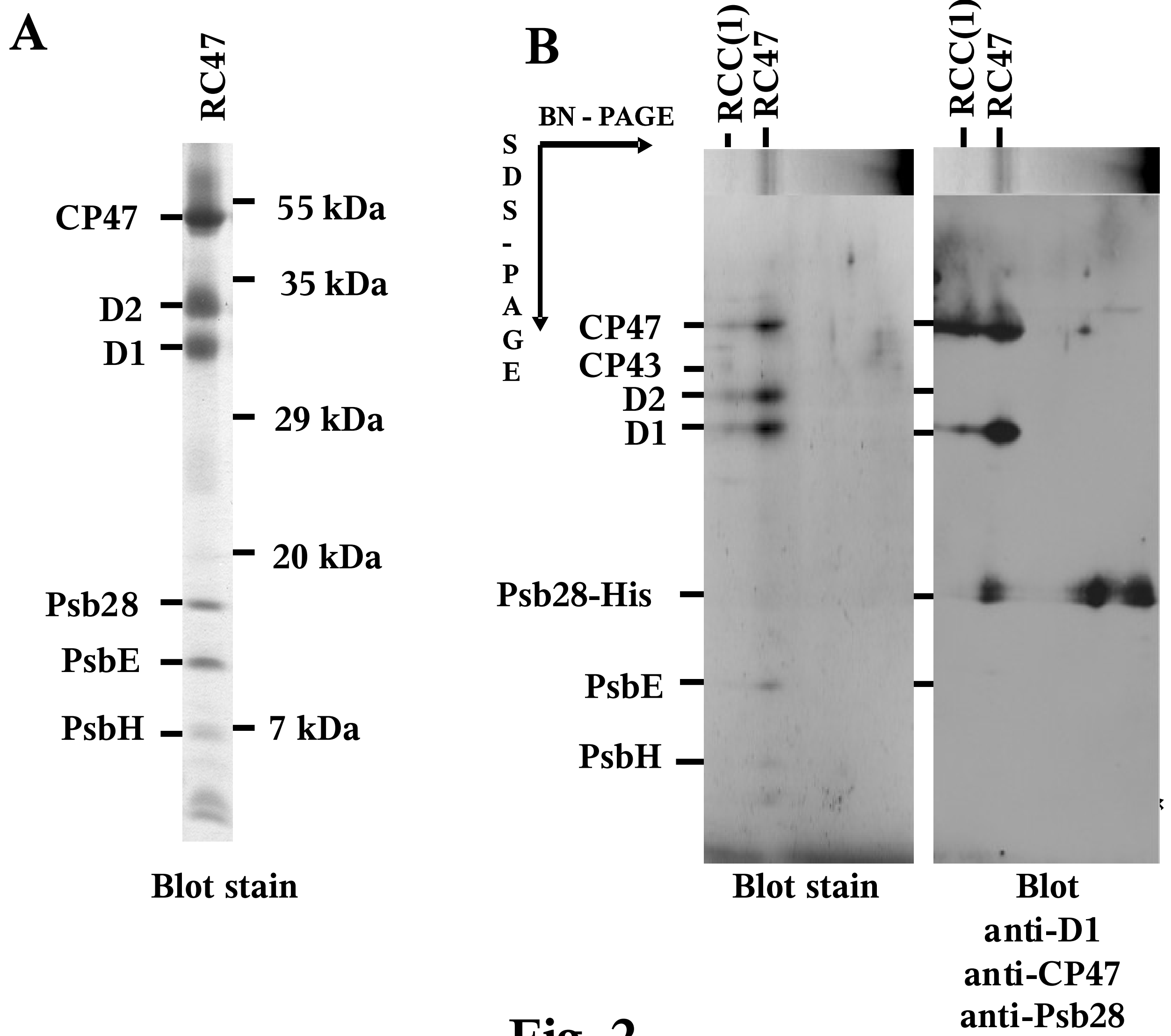

Fig. 2

A

BN - PAGE
SDS - PAGE

WT

PSI(3) RCC(2) PSI(1) RCC(1) RC47 U.P.

PSI
CP47
CP43
D2
D1

1

PSI
CP47
CP43
D2
pD1
iD1
D1

3

Psb28

ΔPsb28

PSI(3) RCC(2) PSI(1) RCC(1) RC47 RCa U.P.

2

Coomassie stain
Autoradiogram
Blot

B

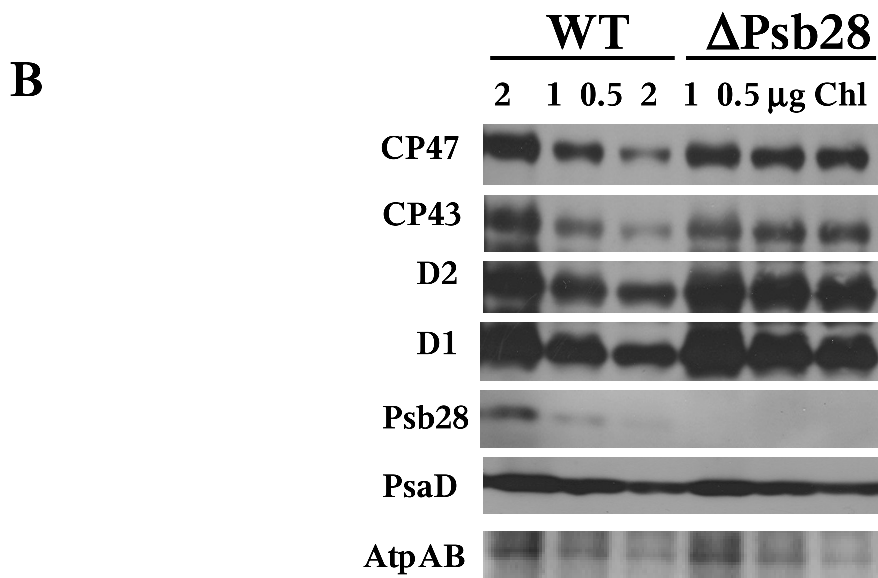

Fig. 3

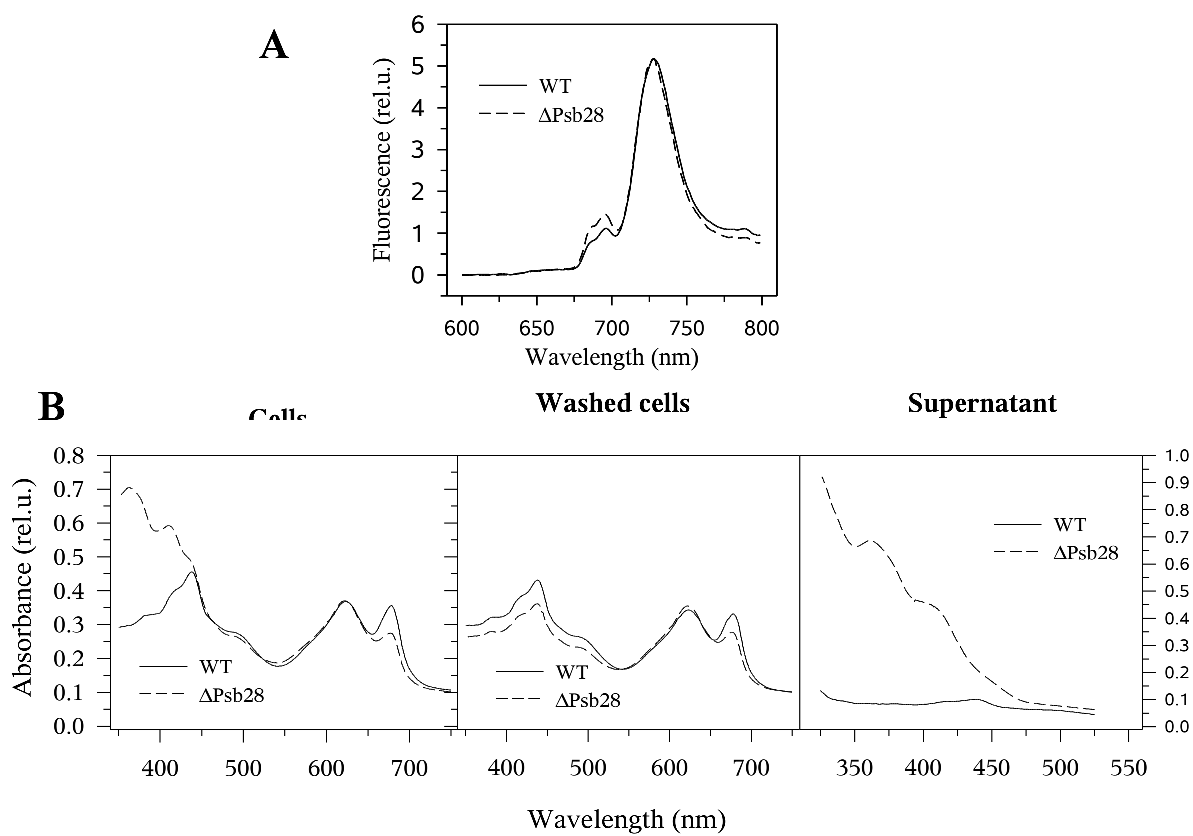

Fig. 4

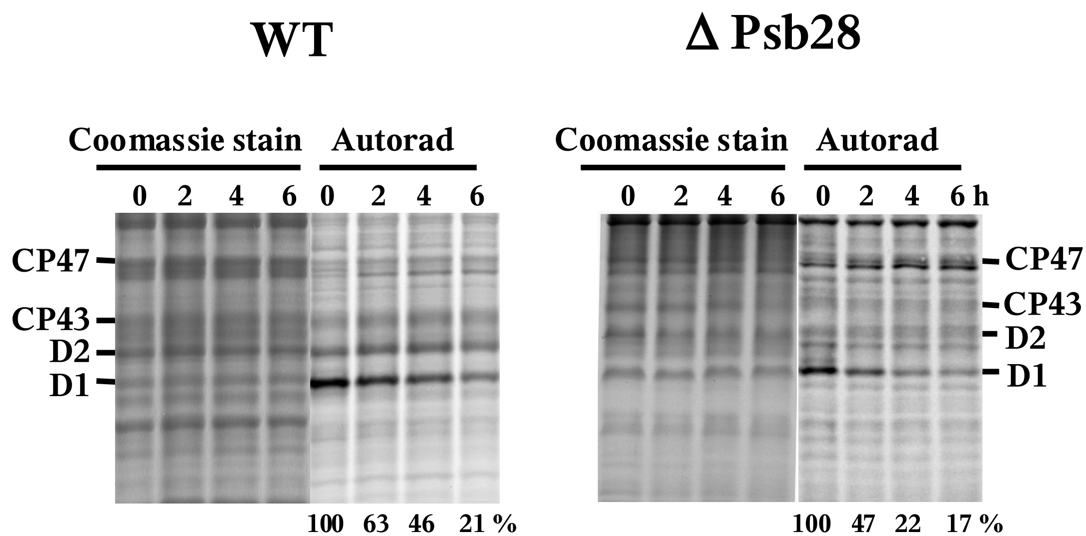

Fig. 5

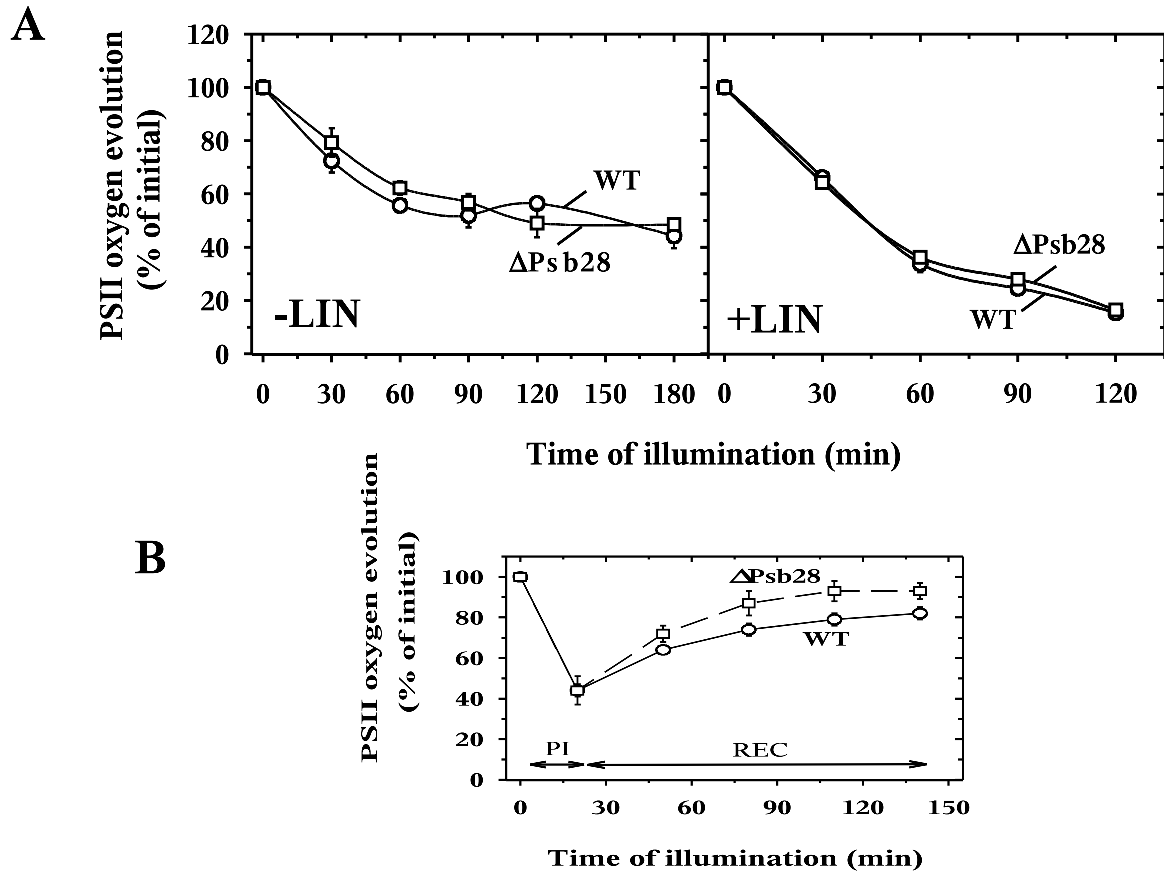

Fig. 6

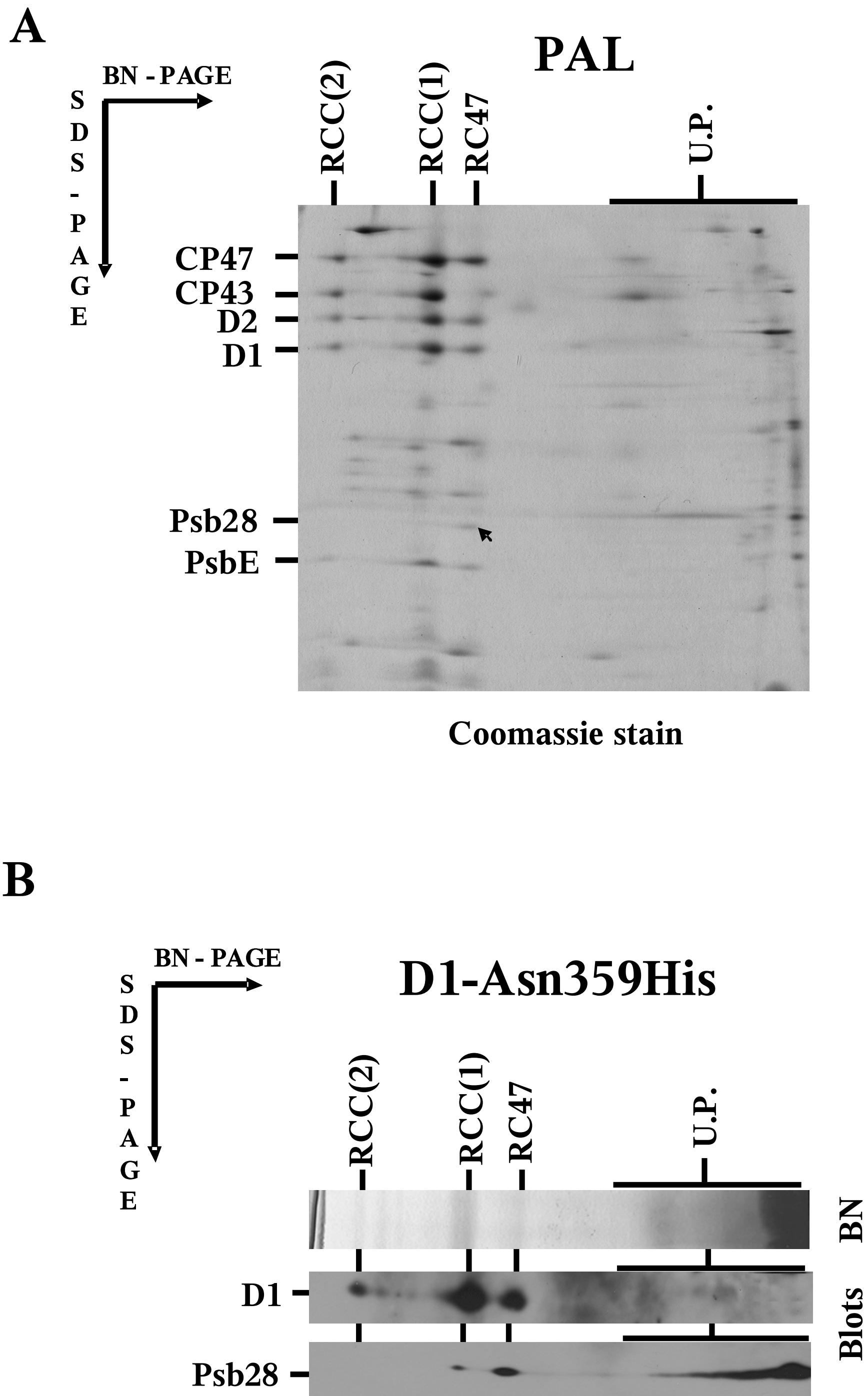

Fig. S1