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 psbA, psbB, psbC, psbDIC/DII, or the psbEFLJ operon. In vivo pulse-chase radiolabeling experiments revealed that mutant cells assembled PSII precomplexes only. In ΔpsbB and ΔpsbB/ΔpsbA, assembly of reaction center cores lacking CP43 and reaction center complexes was detected, respectively. In ΔpsbA, protein subunits CP43, CP47, D2, and cytochrome b$_{559}$ were synthesized, but proteins did not assemble. Similarly, in ΔpsbD/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 ΔpsbEFLJ, 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 psbEFLJ 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) reaction center core complex of higher plants, algae, and cyanobacteria can be subdivided into a heterodimer containing D1 and D2, and 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 phototrophically, 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-
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 psbE and psbF, respectively (32). In plants and cyanobacteria they are part of the psbEFLJ operon and are cotranscribed with PSII subunits L and J. In Chlamydomonas, where psbE is transcribed separately from other PSII genes, D1, D2, and CP47 did not accumulate when psbE was deleted confirming the importance of cytochrome b_{559} for accumulation of PSII (33). Furthermore, recent characterization of psbJ and psbL 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 ΔpsbA, ΔpsbB, ΔpsbC, ΔpsbD, and ΔpsbEFLJ 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 psbEFLJ 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 [³⁵S]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 polycrylamide gel (BN-PAGE). When the buffer front had reached about ½ 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 Na₂CO₃, 2% SDS, and 0.66% 2-mercaptoethanol. Lanes were placed on top of a 12.5% polycrylamide gel containing 4 μl 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 polycrylamide gel containing 7 μl 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 Protein 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 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 polycrylamide gel containing 7 μl 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

**TABLE I**

| Strains | PSII protein missing | PSII oxygen evolution | PSII | Ref. |
|---------|---------------------|----------------------|------|-----|
| WT      | CP43                | +                    | 100  | 60  |
| ΔpsbC   | CP47                | ~                    | <20  | 23  |
| ΔpsbB   | CP47, CP43          | ~                    | <10  | 24  |
| ΔpsbB/C | D1                  | ~                    | 0    | 28  |
| ΔpsbDC  | D2, CP43            | ~                    | 0    | 31  |
| ΔpsbEFLJ| a, β cytochrome b_{559}, PsbL, PsbJ | ~ | 0, 29, 32 |

**Strains and Culture Conditions—*Synechocystis* PCC 6803 mutants and PSII activity**
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 mutants 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. 1A).

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. 1A, 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, CP43, CP47, 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. 1B, 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. 1B 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. 1E, also see Fig. 3 and Refs. 49 and 50). The iD1 form was also found in a reaction center core subcomplex with a molecular mass of 220 kDa termed RC47, which contained CP47, but no CP43 (Fig. 1B). In addition, two complexes with a molecular mass of 110 and 140 kDa were identified which assembled no mature D1 form (Fig. 1D). Because of the radiolabeling of iD1 and identification of D2 in these complexes (Fig. 3D), we called these complexes reaction center complexes and labeled them with a and b, according
to their molecular mass (140 kDa, RCa, and 110 kDa, RCh). In both RCC1 and RC57, 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. 1C), whereas only a minority of the radiolabel accumulated in the dimeric RCC (Fig. 1A, 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 RCh complexes and some D1 accumulated in both complexes (Fig. 1C, 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 RCh complexes (Fig. 1E). 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. 1E). Hence, we concluded that reaction center complexes RCa and RCh 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. 1B, CP47). Radiolabeled CP43 appeared in RCC1 and in two small complexes termed CP43a and CP43b, according to their decreasing molecular mass (Fig. 1B, 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 psbC 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. 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. 2B). 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. 2B). 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 psbC or accumulation of CP43 may be coupled to the expression of psbD or stability of the D2
protein. We therefore tested next whether absence of CP47 in the absence of D1 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. 3A).

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. CP43a and CP43b, with decreasing molecular masses of 70–90 kDa were detectable. Furthermore, ATPase and Rubisco were still 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 psbA expression did not down-regulate expression of psbB, psbC, or psbD or
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/psbD11 Deletion Strain**—In the ΔpsbD/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 blocked although strongly massie stained. Radiolabel incorporation demonstrated that expression of D1 and CP47 was blocked although strongly massie stained.

**Expression of the psbEFLJ Operon Is Selectively Required for psbD Expression and RC Precomplex Assembly**—When *Synechocystis* cells without the psbEFLJ 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. 7B, oblique arrows). Most remarkably, the expression pattern of CP47 and pD1, as well as processing of pD1 is possible. Comparison between protein labeling in the ΔpsbD/C mutant and in the other studied strains showed that in general the synthesis of membrane proteins is reduced in this mutant.

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**Fig. 5. Two-dimensional BN/SDS-PAGE analysis of a Synechocystis psbA triple deletion strain lacking D1.** Thylakoid membrane proteins from ΔpsbA 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 psbDIC/psbD11 double mutant lacking D2 and CP43.** Thylakoid membrane proteins from ΔpsbD/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. 1D, B.

The expression of the psbEFLJ 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 b559 subunit we then analyzed the presence of cytochrome b559 in protein complexes.

**Cytochrome b559 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 b559, 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 b559. In the wild type, the cytochrome subunit was readily found in RCC1 as well as in RC47 (Fig. 8A). 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 psbEFLJ 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 ΔpsbC, the subunit was found in RC47 (Fig. 8B). In ΔpsbE, immunodetection showed its presence in both RCa and RCb complexes (Fig. 8C). In the psbA deletion mutant, the position of cytochrome b559 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. 8D, 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 b559 RC precomplex similar to that found in higher plants (40). The cytochrome band in the 50–70-kDa region was also found in the ΔpsbD/C strain showing that synthesis of cytochrome b559 and accumulation of this complex was independent from psbD expression (Fig. 8E, ΔpsbD/C). In contrast to ΔpsbA, in ΔpsbD/C no partially separated cytochrome subcomplex was detected at higher molecular mass. In the psbEFLJ deletion mutant, almost no accumulation of D1 and D2 was detectable, which confirmed the requirement of cytochrome b559 for biogenesis of PSII (Fig. 8F).
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Role of Cytochrome b559 in the De Novo Assembly of PSII—In barley etioplasts, the formation of an RC assembly precomplex containing D2 and cytochrome b559 was shown to precede PSII assembly (40). In Synechocystis, the synthesis and assembly state of the D1, D2, and cytochrome b559 proteins in the psbB, psbDIC/psbDII, and psbEFLJ deletion strains showed that the presence of cytochrome b559 is a prerequisite for D2 synthesis and accumulation, making it likely that a D2-cytochrome b559 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 ΔpsbB and ΔpsbA strains (Figs. 3D and 8D). 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 b559 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 b559 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 b559 precomplex or whether the protein is cotranslationally associated with the D2-cytochrome b559 precomplex during its synthesis remains open (52) (Fig. 9).

Gel blot analysis showed that in all studied strains (with exception of ΔpsbEFLJ) the α-subunit of cytochrome b559 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 ΔpsbC, 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 b559 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 b559 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
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/H11350 > D2/H11022 > CP43/H11022 > CP47 (53).

**Selective Replacement of D1 Predominantly Occurs in RC47**—In the *psbC* 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 *psbA* 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. 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. 8. Gel blot analysis of *Synechocystis* strains after separation of thylakoid membrane proteins by two-dimensional BN/SDS-PAGE.](#)

**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 CP47 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.
than the majority of the protein. Although, we cannot completely exclude that these complexes represent aggregates because of the absence of their proper PSI binding factors, results for CP43 in the ΔpsbB strain (Fig. 3) and for the iD1 protein in the ΔpsbEFLJ strain indicate that accumulation of the distinct bands represent specific complexes (Fig. 1, 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 unassembled or partially assembled states all four radiolabeled PSI 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 radiolabeled CP43 in RCb but not RCa, more CP43b than CP43a accumulated, and D2 was detected merely in lower Mr bands indicating an assignment of the lower Mr bands to complexes not associated with chlorophylls (data not shown). Also, after 16 h preincubation of WT cells in the presence of 5 μM fluridone, 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 ΔpsbB/C strain, where less RCh 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. Acknowledgments—We thank Wim Vermaas (ΔpsbA, ΔpsbB, and ΔpsbD) and Himadri Pakrasi (ΔpsbEFLJ) for providing Synechocystis mutant strains and Peter Nixon (C-terminal D1 and D2) and Antar Matteo (N-terminal D1) and Roberto Barbato (cytochrome b552) for providing antibodies.

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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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