Interaction of the Periplasmic PratA Factor and the PsbA (D1) Protein during Biogenesis of Photosystem II in Synechocystis sp. PCC 6803*

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The biogenesis of photosynthetic complexes is assisted by a growing number of trans-acting factors in both chloroplasts and cyanobacteria. We have previously shown that the periplasmic PratA factor from Synechocystis sp. PCC 6803 (Synechocystis 6803) is required for adequate C-terminal processing of the PsbA (D1) subunit of photosystem II (PSII) supporting the idea that the early steps in PSII assembly occur at the plasma membrane. Here we report on the molecular analysis of the interaction between PratA and the D1 protein. Both yeast two-hybrid and glutathione S-transferase pulldown assays revealed that PratA binds to the soluble forms of both mature and precursor D1 C-terminal regions. In agreement with that finding, the binding region was mapped to amino acid positions 314–328 of D1 by applying a peptide-scanning approach. Approximately 10–20% of the soluble PratA factor was found to be associated with membranes in a D1-dependent manner. Sucrose density gradient centrifugations allowed the identification of a specific membrane fraction that contains both PratA and D1 and which might represent a transfer and/or connecting region between plasma and thylakoid membrane. Imaging data obtained with enhanced cyan fluorescent protein-labeled D1 protein in wild-type and pratA mutant backgrounds further supported this notion.

Photosystem II (PSII) mediates water oxidation and initiates electron flow during light reactions of photosynthesis in cyanobacteria and in chloroplasts of eukaryotes. Cyanobacterial PSII has been characterized in great detail at the structural level (1–4). However, much less is known about the structural level (1–4). However, much less is known about the stepwise biogenesis of this molecular machine, which contains at least 19 protein subunits as well as a complex set of cofactors (5–7). In the prokaryotic model organism Synechocystis PCC sp. 6803 (hereafter Synechocystis 6803), several proteins have recently been identified that associate transiently with PSII, and inactivation of these leads to severe defects in PSII activity. Such proteins represent good candidates for putative assembly factors.

The protein Srl1471p (OXA1/Alb3/YidC homologous), for instance, has been shown to interact directly with the D1 protein during integration of the latter into the thylakoid membrane (8). The luminal factor YCF48 (HCF136) is required for both efficient assembly and repair of PSII (9, 10), and the 11-kDa lipoprotein Psb27 has been implicated in facilitating assembly of the manganese cluster of PSII (11, 12). Finally, the 22-kDa Psb29 protein is apparently necessary for accurate biogenesis of the inner antennae of PSII (13).

We have previously shown that the tetratricopeptide repeat protein PratA is involved in the biogenesis of PSII: loss of PratA function affects the maturation of the reaction-center protein D1 (14). In almost all photoautotrophic organisms, D1 is synthesized as a precursor with a C-terminal extension that must be removed to allow assembly of the manganese cluster at the luminal side of PSII. In Synechocystis 6803, this extension consists of 16 amino acids, which are processed in a two-step fashion (15, 16). The precise contribution of the extension to photosynthetic performance is still under debate, but recent analyses of site-directed mutants have revealed that it has a photoprotective function (17, 18). Intriguingly, PratA is a periplasmic protein that was previously shown to interact directly with the soluble C-terminal portion of D1 in yeast two-hybrid studies (19, 14, 20). This finding strongly supports the idea that the early steps in photosystem biogenesis in cyanobacteria take place at the plasma membrane and not in the thylakoids (21, 22). However, recent ultrastructural analyses have failed to unambiguously solve the long-standing question of whether or not a direct connection exists between plasma and thylakoid membranes; such a link would establish a continuum between the periplasm and the thylakoid lumen (23, 24).

Here, we report on the molecular details of the D1-PratA interaction. Based on two-hybrid studies in yeast and in vitro assays, we mapped the binding region of PratA on the D1 C terminus. Furthermore, we show that PratA forms part of a complex that also contains the D1 protein. Intriguingly, this complex is apparently not localized to the main thylakoid fraction but is enriched in a specific membrane subcompartment.
**PratA-D1 Complex Formation**

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Analysis**—Protein interaction studies in yeast were performed using the MATCHMAKER LexA system (Clontech, see Ref. 14). Cloning of the PratA gene (slr2048) into the yeast expression vector pG4-5 and of a DNA fragment encoding the last 68 amino acids of the pD1 protein into pEG202 has been described previously (14). The coding sequence for the C-terminal segment of mD1, which lacks the 16-amino acid extension found in pD1, was amplified by PCR and subsequently inserted into pEG202 via primer-derived EcoRI and XhoI restriction sites. Site-directed mutagenesis of the sequence encoding the C-terminal region of D1 was carried out using the QuikChange kit (Stratagene).

**GST Pulldown Assays**—GST pulldown assays were performed with recombinant proteins expressed in *Escherichia coli*. After amplification by PCR, the coding region of the PratA gene, excluding the transit sequence (14), was cloned into the EcoRI and XhoI sites of the expression vector pGEX-4T-1. GST fusion protein was expressed in *E. coli* BL21 cells. His-tagged PratA protein was produced in *E. coli* M15 cells (Qiagen) after PCR amplification of the same region and subsequent insertion into the Sall and Pst1 sites of the vector pQE31. Fragments encoding either the C-terminal segment of pD1 (68 amino acids) or mD1 (52 amino acids) were cloned into the EcoRI and XhoI restriction sites in pGEX-4T-1. GST fusion proteins were bound to glutathione-Sepharose 4B (GE Healthcare) for 3 h. The Sepharose beads were then incubated with a mixture of His-tagged PratA protein and GST protein alone for 1 h with gentle rotation, washed five times with washing buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 1% Tween 20) and finally resuspended in SDS loading buffer. After 10 min at 98 °C samples were subjected to SDS-PAGE and subsequently analyzed by Western blotting.

**Peptide Scanning**—To map the binding site(s) for PratA in the C-terminal portion of D1, pentadecameric peptides were synthesized (jpt Technologies, Berlin), which overlapped by 12 amino acids and covered the C-terminal 68 amino acids of pD1, and aliquots (5 nmol) of each were immobilized on a filter membrane (PepSpots™ membrane, jpt Technologies). Subsequently, the membrane was incubated at 4 °C overnight with 20 μg of His-tagged-PratA protein. Bound PratA was localized using the αPratA antibody (25).

**Membrane Isolation and Solubilization**—For preparation of soluble and non-soluble protein fractions, equal amounts of wild-type and *TD41* cells were harvested and resuspended in 500 μl of breaking buffer (50 mM Tris/HCl, pH 7, 20 mM MgCl₂, 20 mM KCl). Cells were then mechanically disrupted with glass beads (0.25–0.5 mm diameter) in a mini-beadbeater (Glen Mills), using three 20-s bursts. After centrifugation for 1 min at 15,000 × g, the supernatant was loaded onto a 1 M sucrose cushion and centrifuged for 30 min at 94,000 × g. The supernatant containing all soluble proteins was collected, and the pellet with membrane-bound proteins was resuspended in breaking buffer containing 5% Triton X-100. Finally, the protein fractions were subjected to SDS-PAGE and immunoblotting.

Membranes for solubilization assays (50 μg of protein) were prepared as described (26), sedimented by centrifugation and washed twice in 5 mM HEPES, pH 7.6. The samples were resuspended in 20-μl aliquots of HEPES buffer, to which 20 μl of 5 mM HEPES, pH 7.6 (control), 0.2 mM Na₂CO₃, 4 mM urea, or 2 mM NaCl was added. After a 30-min incubation on ice and centrifugation for 15 min with 18,000 × g at 4 °C the supernatants were collected and the pellets were washed twice in HEPES buffer. All samples were then subjected to SDS-PAGE and analyzed by immunoblotting.

**Two-dimensional PAGE**—For blue native (BN)-PAGE, membranes (500 μg of protein (26)) were sedimented by centrifugation (30 min, 15,000 × g, 4 °C) and resuspended in 50 μl of ACA buffer (750 mM ε-aminocaproic acid, 50 mM Bis-Tris, pH 7.0, 0.5 mM EDTA). After addition of freshly prepared β-decylmaltoside (10% w/v) to a final concentration of 1.5% (w/v), membrane proteins were solubilized for 35 min on ice. After removal of insoluble material by centrifugation, 8 μl of 5% Coomassie solution (750 mM ε-aminocaproic acid, 5% Coomassie Brilliant Blue) was added to each supernatant, and the samples were loaded onto a 4.5–12% BN gel. Electrophoresis was performed at 50 V at 4 °C overnight, replacing the blue cathode buffer with fresh, colorless buffer a third of the way through the run, as described previously (27). For the second dimension, a single lane of the BN-PA gel was washed in solubilization buffer (66 mM Na₂CO₃, 2% SDS (w/v), 0.67% β-mercaptoethanol) for 20 min and then placed on top of a 12.5% SDS gel containing 4 mM urea. Electrophoresis was carried out at 20 mA for 30 min and then at 4 mA overnight. Subsequently, gels were stained with Coomassie or electroblotted onto nitrocellulose-membranes and probed with various antibodies.

For first-dimension colorless native (CN)-PAGE, Coomassie dye was omitted from samples and cathode buffer. Soluble proteins (500 μg) were mixed with 0.001% Ponceau S and electrophonically separated as reported before (28).

**Separation of Synechocystis Membranes**—Equal amounts of cells from each strain in the early exponential growth phase were harvested by centrifugation for 10 min, washed with 5 mM Tris buffer, and resuspended in buffer I (10 mM Tris, 1 mM phenylmethylsulfonlfyl fluoride, 600 mM sucrose, 5 mM EDTA, 0.2% lysozyme). The suspension was shaken for 2 h at 30 °C and subsequently washed twice in buffer II (20 mM Tris, 1 mM phenylmethylsulfonlfyl fluoride). Cells were disrupted by passing them twice through a French Press at 1200 p.s.i. DNase I (20 μl) was added, and, after incubation for 15 min at 4 °C, cell debris was sedimented at 4 °C (4500 × g, 10 min). The supernatant was adjusted to 50% sucrose by adding 80% sucrose in 10 mM Tris. A 10-ml sample of this solution was overlaid with 8 ml of 39% sucrose, 6 ml of 30% sucrose, and 8 ml of 10% sucrose (all in 10 mM Tris, 1 mM phenylmethylsulfonlfyl fluoride). After centrifugation at 4 °C for 17 h at 135,000 × g the gradient was fractionated into five fractions containing 10% (I), 30% (II), or 50% (V) sucrose. The part containing 39% sucrose was divided into two separate fractions (III and IV) for obtaining a higher resolution in this region of the gradient. The samples were concentrated via ultrafiltration (Millipore) and then separated by SDS-PAGE, blotted onto nitrocellulose membrane, and finally probed with various antibodies.
Fraction number V was diluted with 5 mM Tris buffer to a sucrose concentration of 20% and then centrifuged at 4 °C on a linear (30% to 60%) sucrose gradient for 17 h at 135,000 × g. After fractionation, proteins were subjected to immunodetection.

Construction of Strains Expressing eCFP-D1 Fusion Proteins and Confocal Microscopy—For tagging the D1 protein with enhanced cyan fluorescent protein (eCFP), both the promoter and the coding region of the psbA2 gene (slr1311) from Synechocystis 6803 were PCR separately amplified from wild-type genomic DNA. The eCFP coding region was amplified from plasmid p2GWC7 (29) and inserted via appropriate primer-derived restriction sites into the N terminus of the plasmid p2GWC7 (29) and inserted via appropriate primer-derived restriction sites into the N terminus of the psbA gene. The fusion gene was then inserted into the Smal site of the conjugation vector pVZ322 (30) giving rise to plasmid N-eCFP-D1. Wild-type and pratA mutant cells were each conjugated with this plasmid by following the protocol of (30). Conjugants were then suspended in 0.3% Gelrite (w/v) (Serva) and applied to coverslips for fluorescence microscopy. Confocal images were acquired with a TCS-SP5 confocal laser scanning system equipped with an inverted microscope (Leica) and an 63× glycerol immersion objective (numerical aperture, 1.3). For the specific detection of eCFP and chlorophyll, excitation wavelengths were set to 480 nm and 670 nm, respectively.

RESULTS

PratA Interacts with the Mature D1 Protein—We have previously shown that the soluble, periplasmic tetratricopeptide repeat protein PratA from Synechocystis 6803 is involved in the biogenesis of PSII (14). The analysis of pulse-labeled proteins had suggested that the C-terminal processing of the reaction-center protein D1 is affected by a pratA mutation. In agreement with this finding, a direct interaction between the soluble C-terminal 68 amino acids of the D1 precursor (pD1) and PratA was documented using a yeast two-hybrid system (14) (Fig. 1).

To test whether PratA binding is specific for the precursor form of the D1 protein, we have now analyzed a version that lacks the C-terminal extension characteristic of the precursor using the same two-hybrid approach in yeast. As shown in Fig. 1A, PratA also recognizes the C-terminal segment of the mature D1 (mD1), suggesting that the 16-amino acid extension present in the precursor is not required for binding of PratA. Instead, the 52 amino acids retained in mD1 appear to contain the crucial determinants that mediate the D1-PratA interaction.

Previous attempts to demonstrate this interaction using in vitro approaches yielded ambiguous results (14). However, optimization of the conditions used for GST pulldown assays has now allowed us to confirm the specific interaction of the D1 protein and PratA also in vitro (Fig. 1B). Sepharose-bound GST alone failed to capture recombinant His-tagged PratA protein. In contrast, matrix-bound GST fused to the C-terminal portion of either the precursor or the mature D1 interacted with recombinant PratA, and PratA could subsequently be eluted from the matrix material under denaturing conditions (Fig. 1B). As a negative control, E. coli proteins from a strain containing no recombinant PratA protein were always analyzed in parallel. No signals were obtained in these cases. Taken together, the data thus strongly suggest that PratA specifically recognizes the C-terminal sequence that is common to pD1 and mD1.

Mapping of the PratA Binding Region in the C-terminal Segment of D1—To determine the one or more regions that mediate this interaction, a peptide scan was performed based on immobilized, pentadecameric peptides with 12-amino acid overlaps and covering the entire C-terminal segment of pD1. Recombinant PratA protein was incubated with the PepSpots™ membrane bearing the whole pep-
tide set, and subsequent decoration with the αPratA antibody revealed that PratA specifically binds to peptide No. 8, corresponding to positions 314–328 of the D1 protein (Fig. 2, A and B). Peptide No. 1 (positions 293–307) was also recognized by PratA, although the signal was less pronounced. We then analyzed mutant versions of the D1 C terminus that lacked either peptide 1 or 8 in the yeast two-hybrid system. Deletion of amino acids 293–307 had only a limited impact on yeast growth, suggesting that these positions make only a minor contribution, if any, to PratA-D1 complex formation (Fig. 2C). In contrast, when residues 314–328 were deleted, no detectable PratA-D1 complex was observed (Fig. 2C). This indicates that the region represented by peptide 8 indeed contains essential determinants that are strictly required for recognition of PratA. It is intriguing that, based on available structural data of crystallized PSII, this D1 region forms an α-helical structure (4). Furthermore, peptides 7 and 9, each of which overlaps peptide 8 by 12 amino acids, exhibited no detectable PratA binding at all, suggesting that the entire helix and/or its left and right borders are required for recognition by PratA. To substantiate this hypothesis, we replaced the threonine at position 316 with a glycine, because alignments of D1 sequences from various organisms revealed that Thr-316 is the only highly conserved amino acid at the left end (Ile-Gly-Thr) of the helix region. Yeast two-hybrid analysis indeed revealed that the T316G mutation is sufficient to prevent PratA binding completely (Fig. 2C).

PratA Forms Part of Two Different Protein Complexes—Because an association with the D1 protein should result in a membrane localization of the otherwise soluble PratA protein, we measured the amounts of PratA present in soluble and membrane fractions of broken Synechocystis 6803 cells (Fig. 3A). In the wild-type, ~10–20% of PratA was found to co-sediment with the membrane fraction in three independent experiments. In contrast, in the triple psbA deletion strain TD41, which fails to accumulate any D1 protein (31), only minute amounts of PratA were found to be associated with membranes, supporting the idea of a PratA-D1 interaction (Fig. 3A). The nature of the membrane association of PratA was examined further by testing the effects of various potential solubilizing agents (Fig. 3B). Treatment of membranes with 1 M NaCl did not release PratA from the membrane, suggesting that non-ionic interactions play a crucial role for its localization. This was confirmed by the finding that 0.1 M Na₂CO₃ or 4 M urea was capable of solubilizing substantial amounts of PratA protein (Fig. 3B).

To analyze the membrane-associated and soluble forms of PratA in more detail, two-dimensional gel electrophoresis of protein subfractions was performed. When the membrane fraction was analyzed by two-dimensional BN-SDS-PAGE, PratA was detected together with unassembled D1 protein in the 70-kDa range (Fig. 4A). In addition, smaller amounts of PratA material formed a faint smear toward higher molecular mass regions (>100 kDa, Fig. 4A). However, various PSII core complexes, such as RCa, RC47, and RCC (32), did not contain any detectable PratA protein. These data are consistent with the idea that a membrane-associated D1-PratA complex forms very early during the biogenesis of PSII. When the TD41 strain was similarly analyzed, no PratA protein was detected in the membrane fraction, again indicating that the membrane association of PratA is solely mediated via the D1 protein (Fig. 4A, compare also Fig. 3B). Two-dimensional CN-SDS-PAGE of soluble proteins from the two strains revealed a different picture (Fig. 4B). In wild-type cells, the soluble PratA form is found in a complex of ~200 kDa. Subsequent analysis of the psbA deletion strain TD41 revealed

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**FIGURE 3. PratA is membrane associated.** A, membrane (M) and soluble (S) protein fractions were prepared from wild-type Synechocystis 6803 (WT) and the psbA deletion strain TD41, and separated by ultracentrifugation, subjected to Western blotting, and incubated with the antibodies indicated on the right margin. B, samples of the membrane fraction from the wild type were treated with the indicated chemicals, and separated into pellet (M) and supernatant (S) fractions by centrifugation. These fractions were then probed with αPratA antibodies.

**FIGURE 4. PratA-containing complexes.** A, membrane fractions obtained from wild-type, pratA, and TD41 strains were solubilized with 1.2% β-dodecylmaltoside, separated by two-dimensional BN-SDS-PAGE, and immunoblotted. PratA and D1 were then localized using the respective antibodies. A representative two-dimensional gel loaded with a wild-type extract and stained with Coomassie is shown at the top. B, similar analysis of soluble proteins, which were separated by two-dimensional CN-SDS-PAGE. The Coomassie-stained gel at the top shows the pattern obtained after separation of a wild-type extract by CN-PAGE.
JOURNAL OF BIOLOGICAL CHEMISTRY

that neither the size nor the amount of this complex is altered in
the absence of the D1 protein (Fig. 4B). This is in striking con-
trast to the behavior of the smaller, membrane-associated com-
plex. Thus, two PratA complexes that differ in size and local-
ization exist in Synechocystis cells. Whether these complexes
are functionally related remains to be elucidated.

Repeated attempts to immunoprecipitate the PratA-D1 com-
plex were initiated to confirm its presence in vivo. How-
ever, presumably due to the low abundance of the complex,
evidence for the presence of neither PratA nor D1 in various
precipitates remained equivocal (data not shown).

The PratA-D1 Complex Is Localized to a Membrane Subcompartmet—All the data presented above strongly sug-
ggest that a PratA-D1 complex is formed in vitro and in vivo. But
is this complex associated with the plasma membrane or the
thylakoid membrane system in Synechocystis 6803? To answer
this question, we employed sucrose density gradient centrifuga-
tion to separate the two membrane types. Initial centrifuga-
tion through a step gradient allowed the partial purification of
the plasma membrane, as judged by the immunodetection of
the plasma membrane marker NrtA (Fig. 5A). However, NrtA
was detectable in all fractions, including that containing the
thylakoid fraction V, whereas D1 was only detectable in
fraction V, even after overexposure of films. We did not further analyze
the PratA-containing fraction II, because the main focus of the work
was on the characterization of the native D1-PratA complex, which
must be localized in the crude thyla-
koid fraction V of the gradient. To

PratA-D1 Complex Formation

fraction V, even after overexposure of films. We did not further analyze the PratA-containing fraction II, because the main focus of the work was on the characterization of the native D1-PratA complex, which must be localized in the crude thylakoid fraction V of the gradient. To further resolve the membrane mixture present in this fraction, it was subjected to a second centrifugation through a linear 30% to 60% sucrose gradient (Fig. 5B). This resulted in a clear separation of PratA (Fig. 5B, fractions 1–6) from the chlorophyll-containing thylakoid membrane fractions, which contained most of the mature mD1 protein (Fig. 5B, fractions 7–14). In contrast to mD1, the precursor pD1 protein exhibited a different distribution on the linear sucrose gradient. Roughly similar amounts of pD1 were detected in the PratA-containing fractions 3–7 and the thylakoid fractions 11–13. The inner antenna protein PsbB was identified only in thylakoid fractions 7–14. However, due to the lower sensitivity of the antibody used, we cannot exclude the possibility that minor amounts of PsbB are also present in fractions 1–6. Hence, the

data strongly suggest that PratA is not associated with thylakoid membranes per se, but rather with a distinct membrane sub-
fraction, which co-migrates with thylakoids during centrifuga-
tion through the step gradient (Fig. 5A). NrtA again showed a
distribution throughout the whole gradient (Fig. 5B). It remains
unclear whether this really reflects the sedimentation of the
plasma membrane or might represent a special feature of NrtA
during sucrose gradient centrifugation. However, no absolute
co-fractionation of PratA and neither NrtA nor PsbB was
observed in these experiments strongly suggesting that PratA
marks a specific membrane subcompartment of Synechocystis
6803 cells. To answer the question whether the PratA form in
fractions 1–6 of the linear gradient indeed represents the mem-
brane-associated one two-dimensional-BN-SDS-PAGE was
carried out on material from fraction 1 (Fig. 5B). The results
demonstrated that indeed the smaller membrane-associated
PratA complex of 70 kDa was present in this fraction (Fig. 5C).

When membranes from the pratA mutant were fractionated in
the same way, a moderate but significant shift of mD1 material
toward the top of the gradient was observed (Fig. 5B, compare
lanes 4–7 from WT and pratA). In contrast, both NrtA and PsbB
exhibited the same sedimentation behaviors irrespective of its
source. The latter finding indicates that only a PSII subfraction
that does not contain appreciable amounts of PsbB is affected by the
pratA mutation. The strongest impact due to the absence of
PratA was detectable for both the amount and the distribution of

FIGURE 5. Cellular sublocalization of PratA. A, total cellular proteins were centrifuged through a sucrose step gradient, fractionated into five portions containing 10% (I), 30% (II), 39% (III and IV), and 50% (V) sucrose and subjected to Western analysis with the indicated antibodies. B, the material from fraction V in A was then centrifuged on a second linear sucrose gradient (30–60%), and fractions were analyzed as in A using the indicated antibodies. C, the material from fraction I at the top of the second gradient was analyzed by two-dimensional BN-SDS-PAGE as in Fig. 4A.
PratA-D1 Complex Formation

![Diagram](image)

**FIGURE 6. Localization of eCFP-tagged D1 protein in Synechocystis 6803.**

A, Western analysis of eCFP-D1 and D1 accumulation in indicated strains by using respective antibodies. The Coomassie-stained gel is shown as loading control. B, wild-type (WT) and pratA cells conjugated with plasmid N-eCFP-D1 (WTN and pratA) and suspended in 0.3% Gelrite were layered on slides, and fluorescence images were taken at excitation wavelengths of 480 nm (eCFP) and 670 nm (chlorophyll). Chlorophyll autofluorescence is shown in red and eCFP fluorescence in blue. The merged images show the distribution of both signals. White scale bars represent 1 μm.

D1 precursor protein. The specific membrane fractions (1–6) showed a strong accumulation of pD1, whereas pD1 was missing in the chlorophyll-containing fractions (7–14). Taken together, these data suggest that, in the pratA mutant, an early intermediate in PSII assembly is at least partially retained in a membrane subfraction with which the wild-type PratA protein would normally associate. Furthermore, the data underline the formerly described pD1-processing phenotype of the pratA mutant (14).

**PratA-mediated D1 Localization**—The data presented in the previous section suggest that the PratA-D1 complex is localized to a special membrane subcompartment, which forms an intermediate between the plasma membrane and the thylakoid membrane systems. This subcompartment could therefore represent a structural connection between the plasma and thylakoid membranes (23). This would in turn imply that biogenesis of the PSII core starts in the plasma membrane, as has previously been postulated (21). If this is true, one may speculate that PratA is involved in some way in the transfer of the D1 protein from the plasma membrane to the thylakoid membrane. To test this possibility, we used conjugation to construct strains that express an N-terminally eCFP-tagged D1 protein, in addition to the endogenous D1 protein, either in a wild-type or pratA genetic background. As depicted in Fig. 6A the D1-eCFP fusion protein is expressed abundantly in the conjugated wild-type (WT) and at a reduced rate in the pratA mutant (pratA) background. In non-conjugated cells the specific eCFP signal was undetectable. D1 protein accumulation was slightly compromised in conjugated lines suggesting that the tagged D1 version competes to some extent with endogenous D1 (Fig. 6A).

The eCFP-tagged D1 protein could be visualized by fluorescence in the wild-type and pratA conjugant lines. As expected from the Western analysis a weaker signal was obtained in pratA. To assess whether the eCFP-D1 fusion protein reaches the thylakoid membrane, we monitored chlorophyll autofluorescence at an emission wavelength of 670 nm. In the wild-type background, the eCFP emission coincided perfectly with the chlorophyll autofluorescence, thus confirming colocalization of the eCFP-D1 fusion protein and the thylakoid membranes (Fig. 6B). This indicates that the eCFP tag does not interfere with insertion of the fusion protein into PSII precomplexes. In the pratA mutant, in contrast, eCFP emission did not colocalize with thylakoids to the same extent. Instead, it was mainly localized to the periphery of cells and did not show complete coincidence with the chlorophyll fluorescence (Fig. 6B). Although the autofluorescence is visible throughout the cell, with the exception of the cytosolic compartment, eCFP in the pratA mutant was restricted to a narrow sphere at the periphery of the cells, which may possibly colocalize with the plasma membrane (Fig. 6B). This may indicate accumulation of D1 fusion protein in the plasma membrane or the membrane subcompartment identified by our cell fractionation studies. As expected, eCFP emission could not be detected in non-conjugated wild-type cells (negative control, Fig. 6B). Taken together, these data provide an additional piece of evidence that the PratA factor might be involved in the transfer of newly synthesized D1 protein from the plasma membrane to the thylakoid membrane system.

**DISCUSSION**

**Interaction of PratA and D1**—We previously suggested that the periplasmic tetratricopeptide repeat protein PratA is involved in the C-terminal processing of the D1 protein. However, here we demonstrate, using the yeast two-hybrid system as well as GST pulldown assays, that the C-terminal extension of the D1 protein is dispensable for PratA recognition. This is consistent with our mapping of the PratA binding site to the α-helix between positions 314–328 (peptide 8, see Fig. 2A) of the mature D1, and with the fact that alteration of the conserved threonine at position 316 of D1 into a glycine completely prevents binding of PratA. Because membrane association of PratA is mediated by non-ionic interactions, it remains to be seen what the precise determinants for D1 recognition by PratA are. Nevertheless, one can conclude from the peptide scanning data that, in addition to Thr-316, one or more amino acids from the opposite end of the helix (positions 326–328) are also required for efficient PratA binding.

In light of these new findings, the D1-processing phenotype of the pratA mutant might be interpretable simply as a secondary effect of a perturbation in the PSII assembly process. For instance, C-terminal D1 maturation has been shown also to be affected in mutants lacking distinct subunits of PSII, i.e. PsbH, PsbB, or PsbEFLJ (33). Furthermore, yeast two-hybrid analyses have provided no evidence for a direct interaction of PratA with the C-terminal D1 protease CtpA (data not shown). Thus, the precise molecular function of PratA remains to be dissected.

**PratA Forms Part of at Least Two Protein Complexes**—Two-dimensional BN-SDS-PAGE revealed that the membrane-associated and soluble forms of PratA are found in complexes that differ significantly in size. The soluble 200-kDa complex is not affected by the absence of the D1 protein. In contrast, formation of the 70-kDa membrane-associated complex is dependent on D1, and its relatively small size suggests that it represents a very early intermediate in PSII assembly that contains at least the D1 protein in addition to PratA. The *Synechocystis* homolog of HCF136/YCF48 has also recently been shown to interact with the D1 precursor protein and the C-terminal processing intermediate ID1, but not with the mature D1 protein, during
the early phase of assembly (10). However, no direct interaction between HCF136 and PratA could be detected in the yeast two-hybrid system (data not shown), suggesting that the two factors may interact only transiently or not at all.

Localization of the PratA-D1 Complex—Based on cell fractionation experiments it had earlier been proposed that the first steps in de novo assembly of PSII and PS I take place at the cytoplasmic membrane and not on the thylakoids of Synechocystis 6803 (21, 22). For PSII, the D1 and D2 proteins, as well as cytochrome b559 subunits, were shown to be present in plasma membrane fractions, whereas the PsbB and PsbC subunits were exclusively detected in thylakoids (21, 34). Furthermore, the PratA subunits in the cytoplasmic membrane accumulated in inside-out rather than right-side-out vesicles, indicating a heterogeneous organization of the plasma membrane (35). This suggests that discrete regions of the plasma membrane harbor sites at which assembly of PSII is initiated (35). Interestingly, in chloroplasts of the green alga Chlamydomonas reinhardtii, distinct membrane subfractions around the pyrenoid have been identified as being sites of de novo assembly of PSII, whereas D1 repair synthesis was found to occur throughout the entire thylakoid membrane system (36).

Here, using a two-step cell fractionation procedure, we identified a membrane subfraction in which PratA specifically accumulates together with substantial amounts of the precursor pD1 protein. PsbB was not detected in appreciable amounts in these fractions, which is consistent with the abovementioned data suggesting that PsbB is absent from plasma membranes (21, 34). Therefore, it is obviously tempting to speculate that this subfraction represents membrane regions at which initial steps in PSII assembly occur. This idea is further supported by the finding that pD1 accumulation significantly increases in these fractions in the absence of PratA. Extensive additional work will be required to characterize this cellular compartment in greater detail. It will be important to test how its proteomic composition is organized and whether other factors involved in thylakoid membrane biogenesis accumulate there.

Localization of an eCFP-tagged D1 protein at the cell periphery in a pratA”-genetic background further supports the idea that PratA is involved in processes related to the preassembly of PSII core complexes at specific sites within the plasma membrane and/or in the transfer of complexes from these sites to the thylakoid membrane. However, the localization effect was not very pronounced, probably due to the fact that the pratA mutation leads not to a fully fledged PSII”-phenotype but only to a reduction of PSII levels to 25% of the wild-type value. Hence, pratA” cells can still grow photoautotrophically, and all steps in photosystem biogenesis, including membrane transfer processes, can in principle occur, albeit at reduced rates. Future work will try to establish an inducible imaging system that will allow us to monitor D1-eCFP fluorescence with high temporal resolution.

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