PratA, a Periplasmic Tetratricopeptide Repeat Protein Involved in Biogenesis of Photosystem II in *Synechocystis* sp. PCC 6803*

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The light reactions of oxygenic photosynthesis are mediated by multisubunit pigment-protein complexes situated within the specialized thylakoid membrane system. The biogenesis of these complexes is regulated by trans-acting factors that affect the expression of the respective subunit genes and/or the assembly of their products. Here we report on the analysis of the *pratA* gene from the cyanobacterium *Synechocystis* sp. PCC 6803 that encodes a periplasmic tetratricopeptide repeat protein of formerly unknown function. Targeted inactivation of *pratA* resulted in drastically reduced photosystem II (PSII) content. Protein pulse labeling experiments of PSII subunits indicated that the C-terminal processing of the precursor of the reaction center protein D1 is compromised in the *pratA* mutant. Moreover, a direct interaction of PratA and precursor D1 was demonstrated by yeast two-hybrid analyses. This suggests that PratA represents a factor facilitating D1 maturation via the endoprotease CtpA. The periplasmic localization of PratA supports a model that predicts the initial steps of PSII biogenesis to occur at the plasma membrane of cyanobacterial cells.

The light-driven reactions of photosynthesis take place at a specialized membrane system, the thylakoids, that contains the essential protein complexes mediating photosynthetic electron transport, which include photosystem II (PSII), 1 the cyt$b^f$ complex, PSI, and the ATPase. Although the structural and functional features of these complexes have been analyzed in great detail, to date relatively little is known about their regulated biogenesis during cell development.

Recent work on vascular plants, eukaryotic algae, and cyanobacteria revealed that numerous regulatory factors exist that are involved in the expression of genes encoding subunits of the photosynthetic membrane complexes and/or their assembly (1, 2). These factors apparently constitute an intracellular communication system that mediates the controlled biogenesis of thylakoids. Among these, one subgroup is represented by so-called tetratricopeptide repeat (TPR) proteins, which share a degenerate protein-protein interaction motif consisting of multiple repeats of 34 amino acids (3, 4). For instance, TPR motifs have been found in factors that are involved in the stabilization of chloroplast mRNAs (5–7), whereas the chloroplast TPR protein Ycf3 is involved in the assembly of PSI (8, 9).

In cyanobacteria, the structurally related Ycfd7 protein was also shown to affect PSI levels (10). Furthermore, the open reading frame (ORF) *slr2048* from *Synechocystis* sp. PCC 6803 (the *Synechocystis* 6803) encodes a TPR protein that is required for light-activated heterotrophic growth (11). These data suggest that the TPR motif might play an important role in the organization/function of regulatory units in both cyanobacteria and chloroplasts of eukaryotic cells.

In addition to the structure and function of this regulatory machinery, the localization of the different biogenesis steps remains an intriguing, still unresolved matter. Current evidence supports a model of an intraorganellar vesicle transport system for chloroplasts (12, 13). The idea of a specialized region where the biogenesis of photosynthetic membrane complexes takes place and that is spatially separated from the thylakoid membrane was further strongly supported by the recent observation that significant amounts of some PSI and PSII subunits are detectable in the plasma membrane of the cyanobacterium *Synechocystis* 6803 where they form chlorophyll-containing precomplexes (14). These data led to the hypothesis that the initial steps of cyanobacterial photosystem assembly may occur at the plasma membrane and not at the thylakoids (14).

One of these complexes, namely PSI, contains more than 25 subunits, which assemble in a highly coordinated fashion. The initial steps of this assembly include the formation of a dimer of the reaction center proteins D2 and D1 and the subsequent binding of Cyth559, PsbI, CP47, and CP43 (15). Much attention has been focused on the D1 protein (16), which is synthesized as a precursor (pD1) with an extension of 8–16 amino acids at its C terminus. The removal of this extension by a specific endoprotease called CtpA (C-terminal processing protease) represents an essential step of PSI biogenesis (17). In *ctpA* mutants, the unprocessed pD1 precursor, though it is incorporated into a PSI complex, impairs the assembly of the manganese cluster of the oxygen-evolving complex of PSI (18, 19), leading to a photosynthesis-deficient phenotype. Because site-directed mutants of the corresponding *psbA* gene lacking the C-terminal extension were shown to grow at wild-type rates (20), the precise function of the C-terminal extension still remains elusive. However, long-term growth of mutant and wild-type strains of *Synechocystis* 6803 in mixed culture conditions indicated that the C-terminal extension might provide a selective advantage on cells, possibly by protecting D1 during integration into the membrane or PSI (21).

In this report, we have shown that ORF *slr2048* from Syn...
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echo gyrus 6803 encodes a periplasmic TPR protein named PratA (processing associated TPR protein) that is involved in the C-terminal processing of the D1 protein. The finding that PratA directly interacts with pD1 supports the recently proposed model of a plasma membrane-localized biogenesis of cyanobacterial photosystems (14).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Synechocystis sp. PCC 6803 wild-type and mutant strains were grown on solid or liquid BG 11 medium (22) at 30 °C in a continuous photon irradiance of 100 μE m⁻² s⁻¹.

Plasmid Constructions, Transformations, and Complementation—The PratA gene was cloned after its PCR amplification with oligonucleotides 2048–5, CCATCGGAACGGTCCTTGAG, and 2048–3, CTA-GAGATTATACGCTT, and insertion of the resulting fragment into the Bluescript KS vector (Stratagene). A XbaI fragment from plasmid pBSL6 (23) containing a kanamycin resistance cartridge was inserted into the single NheI site within the mutant, the pratA gene including its own promoter was PCR-amplified with oligonucleotides 2048a, CCGAAGGTCGCTGACCTGCA, and 2048b, GCCAGCACAGCTTCTGTGACAGC, and cloned into the single XhoI site within the vector pYZ321 that is capable of autonomous replication in Synechocystis 6803 (24). Conjugal transfer of this construct into pratA was performed as described (24).

Southern, Northern, and Western Blot Analysis—Total cyanobacterial DNA was isolated from 50-ml cultures by using the Qiagen DNeasy plant kit. Southern blot analysis was carried out using standard protocols. Isolation of total RNA, electrophoresis, and gel blotting was performed as described (25). Pigment content was determined with a Beckmann DU 7400 spectrophotometer. The gels were stained with Coomassie Blue R, dried, and exposed to phosphorimaging plates (Fuji Photo Film GmbH). The imaging plates were read out with a FLA3000 image reader and quantified with the supplied AIDA software (raytest Isopenmessgeräte GmbH).

For the measurement of chlorophyll concentrations, cells were sedimented by centrifugation and extracted with 100% methanol. The concentration of chlorophyll was calculated from the absorbance values at the extract at 666 and 720 nm (28).

Yeast Two-hybrid Analysis—For the two-hybrid analysis, the system MATCHMAKER LexA (Clontech), with the Saccharomyces cerevisiae strain EGY48 (MATα, ura3, trp1, his3, lexAop(x6)-LEU2) transformed with the plasmid pSH18–34 (29), and the plasmids pEG202 and pG4–5 (30) were used. The PratA gene fragment was amplified by the oligonucleotides TH2048a, AAATTCGAGATTTACGCTACCTG, and TH2048b, CTCGAGCTAGATTACGCTTTCCTTG, and cloned into the yeast shuttle vector pG4–5 via the oligonucleotide-derived restriction sites EcoRI and XhoI. This construct did not contain the signal peptide sequence for localization in the periplasm. The pD1 C terminus was amplified with the oligonucleotides ThpsbAa, GAATTCATGAATTTACGCTACCT, and ThpsbAb, CTCGAGCTAGATTACGCTTTCCTTG, and cloned into the yeast shuttle vector pG4–5 via the oligonucleotide-derived restriction sites EcoRI and XhoI. The pD1 C terminus comprises 68 amino acids, including its 16-amino acid extension.

RESULTS

Generation and Characterization of a pratA Mutant—Computer-assisted similarity searches revealed that the genome of Synechocystis 6803 harbors a total of 22 ORFs encoding putative TPR proteins. During the course of a systematic mutagenesis study of these ORFs, we analyzed slr2048, which encodes a protein of 398 amino acids that is capable of forming nine TPR repeats. Based on this study, the gene was named PratA (Fig. 1A). Interestingly, a transit sequence was predicted at the N terminus of the derived protein that would allow secretion of the protein via the Sec system (Fig. 1A). Indeed, PratA was identified within the periplasm of Synechocystis 6803 by Fulda et al. (31) during a proteomic analysis.

We disrupted the cloned PratA gene by inserting a kanamy-cin resistance cassette into the single NheI site 379 bp downstream of its translation start codon (Fig. 1B). After subsequent transformation of wild-type cells with this construct, resulting transformants were tested for complete segregation of the mutation by applying Southern blot hybridizations (Fig. 1C) and PCR analyses (data not shown). Northern analysis confirmed that no PratA mRNA was present in the segregated pratA mutant strain (Fig. 2A). Moreover, the detection of only one single signal at 1,300 nucleotides indicated a monocistronic transcription of the gene, because cotranscription with either of the adjacent reading frames, slr2047 or slr2049, would have resulted in transcripts with a minimum size of 2,400 or 1,900 nucleotides, respectively. Thus, it is likely that disruption of the PratA gene has no polar effects on the expression of these genes. The absence of the PratA protein in the mutant was confirmed by Western analysis using an oPratA antiserum raised against the recombinant protein expressed in E. coli (Fig. 2B). Finally, the periplasmic localization of PratA was verified after analyzing cellular subfractions of Synechocystis 6803. As shown in Fig. 2B, a signal at the expected size of 35 kDa was detected in proteins from total cells and in the periplasmic subfraction (Fig. 2B, T and P). No signal was obtained in the protein fraction from cells that had been separated from their periplasm (Fig. 2B, −P). In contrast, the cytoplasmic ribosomal protein S1 was found in the −P but not in the P fraction, indicating that the periplasmic proteins are free of substantial cytoplasmic contaminants. Taken together, these data demonstrate that the vast majority of PratA is located in the periplasm.

The photoautotrophic growth rate of pratA was reduced by more than 4-fold when compared with the wild-type, whereas the chlorophyll content was found to be similar in both strains
after comparative analysis of absorption spectra (data not shown). When fluorescence emission spectra at low temperature (77 K) from both wild-type and pratA were measured, a drastic decrease of chlorophyll emission peaks from PSII at 685 and 695 nm was observed in the mutant, suggesting a lower relative PSII content (Fig. 3). This reduced PSII amount was confirmed by the finding that the ratio of maximum and minimum chlorophyll fluorescence (\(F_{M}/F_{O}\)) was significantly reduced in pratA compared with the wild-type (data not shown). Moreover, photosynthetic oxygen production in pratA was reduced to 25% of that of the wild-type rate, again suggesting a considerable effect on PSII content/activity.

To calculate the PSII amount in pratA, Western analysis was performed. As shown in Fig. 4A, the steady state levels of the PSII subunits PsbB, D1, and PsbO in pratA were reduced to \(\sim 45, 15, \) and 10% of that of the wild-type amount, respectively. This suggests a more direct effect of PratA on D1 and PsbO accumulation. In contrast, both the level of the PSI subunit PsaD and the Rubisco enzyme were not or were only slightly affected, indicating that only the amount of PSII is affected in the mutant. In chloroplasts of eukaryotic algae and vascular plants, TPR proteins have been shown to be required for gene-specific RNA stabilization processes. Thus, we tested whether the RNA levels of various genes encoding PSII subunits are compromised in pratA (Fig. 4B). Obviously, this was not the case for either psbA and psbO mRNAs or for psbB, psbC, and psbD transcripts. These data allow the conclusion that PratA functions in the synthesis and/or the stability of PSII subunits.

PratA Is Involved in D1 Maturation—To distinguish between these two possibilities of PratA function, protein pulse-labeling experiments with radioactive [\(^{35}\)S] methionine were carried out. Radiolabeled proteins were first separated by BN-PAGE to resolve native protein complexes and then, in a second dimension, by SDS-PAGE to elucidate their subunit composition (Fig. 5A). The identity of different proteins was determined through comparison of two-dimensional-BN/
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SDS-PAGE analyses of pulse-labeling experiments using deletion mutants of the PSII subunits D1, D2, CP43, and CP47. During pulse labeling of Synechocystis 6803 proteins, PSII subunits and especially the reaction center protein D1 were predominantly labeled independent of the growth conditions used (33). This reflects the active synthesis of PSII and highlights in particular the D1 protein, which is subject to extensive photo damage and, thus, must be constantly repaired via the PSII repair cycle. In wild-type cells, several different intermediates of the PSII assembly process could be visualized. Both the precursor and the mature D1 protein were detected in the early PSII intermediates RCa, RCh, and RC47 (Fig. 5A), whereas the PSII monomer RCC (1) and bigger complexes contained the C-terminal-processed, mature D1 form alone. In the mutant pratA, the synthesis rates of the PSII components were the same as those of the wild-type, indicating that the mutation affects the stability and not the synthesis of the PSII complex. Furthermore, in pratA PSII appears to be assembled because most intermediate PSII complexes were detectable except for the early precomplexes RCa and RCh (Fig. 5A). However, the main difference between wild-type and pratA was the almost complete absence of mature D1 protein in the mutant. This was verified by performing one-dimensional gel electrophoresis of pulse-labeled proteins (Fig. 5B).

As a control, the strain repratA was generated in which, via conjugation, the wild-type PratA gene including its promoter was reintroduced into the mutant on an autonomously replying plasmid (24). The resulting revertant displayed a rescued phenotype with a photoautotrophic growth rate nearly at the wild-type level. In addition, 77-K fluorescence emission spectra indicated a significantly higher PSII/Psi ratio in repratA compared with the mutant (Fig. 3). The incomplete restoration of the wild-type phenotype is probably a result of the extrachromosomal expression of PratA in the revertant.

Consistent with the restored phenotype, efficient C-terminal D1 processing occurred in repratA (Fig. 5B). The ratio of D1 maturation, though, appeared to be slightly reduced. In contrast to the wild-type, in the intermediate PSII monomer most D1 was still present in the precursor form, which was processed only during later PSII assembly steps (Fig. 5A). Taken together, the pulse-labeling data indicate that the PratA protein is involved in the C-terminal processing of the D1 protein. As a consequence, the water-splitting apparatus would not be able to correctly dock to PSII, thereby explaining the observed phenotype of the pratA mutant.

PratA Interacts with the C Terminus of D1—To this end, the obtained results direct us to the question whether PratA interacts directly with the D1 protein and/or the D1 C-terminal protease CtpA. To test this, we applied a yeast two-hybrid analysis that is based on the LexA system. PCR fragments containing the PratA and CtpA genes as well as a fragment encoding the C-terminal 68 amino acids of pD1 were cloned into shuttle vectors pEG202 or pJG4–5. This resulted in translational fusions of the proteins with the LexA-DNA binding domain or the B42 activation domain, respectively. It is important to note that the cloned pD1 C terminus did not harbor any hydrophobic transmembrane regions that could have possibly disturbed the two-hybrid assay by affecting nuclear import of the fusion protein in the yeast system. As shown in Fig. 6, indeed, an interaction between PratA fused to the activation domain and the pD1 C terminus fused to the DNA binding domain was detected by analyzing the expression of the lacZ and LEU2 reporter genes. No reporter gene activity was observed in a negative control experiment in which the constructs were assayed alone (Fig. 6). This indicated that both PratA and pD1 are required for complex formation.

A putative interaction of PratA with the protease CtpA could not be analyzed in this system, because CtpA expressed in yeast was shown to impair the import of fusion proteins into the nucleus of yeast cells where reporter gene activity is regulated (data not shown). This might be because CtpA, though its nature appears hydrophilic, might be membrane-associated. Indeed, it has been reported to be tightly associated with the plasma membrane in Synechocystis 6803 (14). Hence, future work will focus on the establishment of alternative two-hybrid systems that should allow the analysis of membrane proteins.

**FIG. 5. In vivo pulse labeling of proteins.** Isolated and solubilized membranes were separated by (A) two-dimensional-BN/SDS-PAGE or (B) one-dimensional SDS-PAGE, and pulse-radio-labeled proteins were visualized by autoradiography. Different PSII subunits are marked at the left margins. Three non-characterized PSII assembly intermediates between RCC(1) and RCC(2) are marked by asterisks. Sizes of protein standards are given in kDa. RCa and RCh, reaction center complexes a and b, RC47, reaction center complex with CP47, RCC(1) and RCC(2), monomeric and dimeric reaction center core complexes, respectively.

**FIG. 6. PratA-pD1 interaction in a yeast two-hybrid assay.** Three independent transformants expressing the fusion proteins indicated at the right margin were analyzed for LEU2 reporter gene activation by growth on medium containing no leucine (A) or lacZ reporter gene activity by growth on medium containing 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) (B).

(34) to answer the question whether or not PratA also recognizes CtpA. Nevertheless, the sensitive two-hybrid LexA system enabled us to identify a direct interaction of the TPR protein PratA with the C terminus of the PSII reaction center protein pD1.

**DISCUSSION**

In this study, we report on the characterization of the ORF slr2048 from *Synechocystis* 6803, which encodes the periplasmic TPR protein PratA. The insertion of a kanamycin resistance cassette into the PratA gene resulted in a significant reduction of the PSII content in the mutant and an apparent absence of the C-terminal processing of the pD1 protein. This phenotype resembles the one of a CtpA deletion mutant (ΔctpA), which contains no C-terminal processing D1 protease CtpA (17). However, in contrast to ΔctpA, pratA was still able to grow photoautotrophically though with a 4-fold reduced rate. This might explain why no significant accumulation of pD1 was detectable in pratA in Western analysis, but only in pulse-labeling experiments (Fig. 4A). Because N-terminal parts of the PratA gene providing partial activity might still be expressed in the mutant, we recently constructed a site-directed, completely segregated mutant in which the complete ORF slr2048 is deleted. In this mutant, the photoautotrophic growth rate incurred even a 10-fold reduction when compared with the wild-type, but residual photosynthetic activity was still observed. This suggests that PratA is involved, but not mandatory, for D1 maturation. Most likely, it facilitates the CtpA-mediated processing event. Interestingly, such an assisting, additional component for pD1 processing has recently been proposed for spinach chloroplasts, based on the finding that CtpA exhibits a relatively weak affinity to its substrate in *vitro* (35). Thus, it is tempting to speculate whether PratA might represent an assisting factor in *Synechocystis* 6803.

Computer-assisted BLASTP homology searches revealed several ORFs with similarity to PratA in various cyanobacteria. Among these, ORF trs2360 encoding a hypothetical TPR protein from *Thermosynechococcus elongatus* BP-1 and a hypothetical ORF from *Trichodesmium erythraeum* IMS 101 (ZP 00072573) showed the highest alignment scores of 138 and 137, respectively. In *Arabidopsis thaliana*, PratA shares the highest similarity (alignment score of 92) with an O-linked N-acetylgalactosamine transferase (OGT) At3g04240. However, the homologous region is restricted to the N-terminal TPR domain of the enzyme and not to its C-terminal transferase domain (36), thus indicating that PratA is not an OGT. The absence of an obvious PratA homologue in higher plants might reflect the difference in the molecular details of the processing reaction between these organisms: In *Synechocystis* 6803, the removal of the C-terminal extension of 16 amino acids has been shown to be a sequential two-step proteolytic process with a transient accumulation of a processing intermediate (37). In contrast, the 9 amino acids comprising the C-terminal extension of pD1 from higher plants is excised by a single proteolytic step. However, because the TPR motif is highly degenerate (4), further reliable conclusions concerning PratA homologues in other organisms can only be drawn from functional analysis.

The two-hybrid data strongly suggest that PratA directly interacts with the C terminus of pD1, most likely through its TPR domain. Repeated attempts to detect this complex formation in *vitro* by applying Far Western or pulldown experiments with recombinant PratA and pD1 proteins were not conclusive. Most probably this reflects the short-lived character of the PratA-pD1 interaction, a feature that one would expect when the rapid C-terminal processing of pD1 in the wild-type is taken into account.

However, even a transient interaction indicates that the periplasm-located PratA (31) directly contacts pD1, implying that PratA must be present in close spatial vicinity to pD1 within the cell. Interestingly, it has been recently hypothesized that the initial steps of photosystem biogenesis take place at the plasma membrane of *Synechocystis* 6803 (14). Consistent with this is the finding that, by immunological means, the D1 protease CtpA has been detected exclusively in the plasma membrane and not in the thylakoids (14). These data would favor a scenario in which newly synthesized pD1 is inserted into the plasma membrane where it assembles into precomplexes. The C terminus of pD1 would be oriented to the periplasm and processed by CtpA with the assistance of the periplasmic PratA. The preformed PSII complexes would then migrate to the thylakoids by lateral membrane fusion or membrane vesicle transport (32). In this model, a still unresolved matter concerns the maturation of D1 protein that is inserted into pre-existing PSII complexes during the PSII repair cycle. Analogous to the situation in chloroplasts from eukaryotes, one would expect that this process takes place at the cyanobacterial thylakoid membrane and not the plasma membrane. Hence, a D1 maturation pathway at the thylakoids might exist that operates independently from the periplasmic PratA factor. The existence of an additional, spatially separated D1 processing machinery might explain why the inactivation of PratA still allows residual photosynthetic activity and, thus, D1 maturation.

Of course, the proposed model requires further confirmation. Nevertheless, we succeeded in providing the first genetic evidence that a periplasmic factor is involved in PSII biogenesis. Furthermore, our data identify the TPR protein, PratA, that is involved in the C-terminal processing of the D1 reaction center protein of PSII. Future work will be focused on the elucidation of the precise working mode of PratA and, concurrently, on the spatial organization of thylakoid membrane biogenesis.

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**REFERENCES**


