Evidence that D1 Processing Is Required for Manganese Binding and Extrinsic Protein Assembly into Photosystem II*

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Photosystem II (PSII),1 a multisubunit protein complex localized to the thylakoid membranes of cyanobacteria and chloroplasts, performs a light-driven electron transfer from water to plastoquinones, generating molecular oxygen as a byproduct (1). Cyanobacterial PSII consists of more than 20 subunits to plastoquinones, generating molecular oxygen as a byproduct (2, 3). Recent structural studies (4–6) have greatly advanced our knowledge of the arrangement of the components within the functional complex. In particular, new details on the structure of the tetra-manganese-calcium cluster of the oxygen-evolving complex have given key insights into the mechanism of the water oxidation reaction (6).

Despite these advances, these static structures are not adequate to understand the dynamic nature of the assembly and turn-over of the PSII complex. The structural complexity of PSII requires precise and regulated assembly, yet the PSII biogenesis pathway is poorly understood and many questions still remain as to how the components are assembled into a functional PSII complex. Furthermore, PSII assembly occurs frequently, because the PSII complex is rapidly turned over even under normal conditions (7). As a consequence of the electron transfer reactions, the D1 protein is irreversibly damaged, removed from the PSII complex, and replaced with a newly synthesized D1 protein (8). Thus, PSII complexes can be generated from either newly synthesized components or from partially disassembled complexes.

During PSII biogenesis, several events define the assembly of the oxygen-evolving complex, namely D1 processing, manganese cluster assembly, and extrinsic protein association. The D1 protein is synthesized in a precursor form (pD1) containing a C-terminal extension of 16 amino acids in cyanobacteria (9) and 8–9 amino acids in eukaryotes (10, 11). The pD1 protein is co-translationally inserted into the thylakoid membrane, it associates with other PSII membrane components, and is cleaved after Ala-344 by CtpA, a C-terminal processing protease, to yield the mature D1 protein (12, 13). In addition, the inorganic cluster of manganese and calcium is assembled and several extrinsic proteins (PsbO, PsbU, and PsbV) associate with the luminal side of the complex. However, the temporal order of these events during PSII assembly remains poorly understood.

Despite numerous studies with D1 processing mutants, it has been difficult to characterize PSII complexes from these mutants with respect to their manganese content and polypeptide compositions. Studies of D1 processing mutants have revealed that processing of pD1 is essential for the assembly of a functional manganese cluster. However, the exact number of manganese atoms associated with pD1-containing PSII complexes has been the subject of debate (12–15). Manganese measurements of membranes of the Synechocystis sp. strain 6803 (2, 3, 16). EPR studies of the LF1 mutant revealed the lack of the S2 multiline signal suggesting that the LF1 mutant lacks the redox active manganese cluster of the oxygen-evolving complex (17). Analysis of thylakoid membranes from this mutant showed reduced binding of the 23-kDa

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1 The abbreviations used are: PSII, photosystem II; pD1, precursor D1; TES, 2-(2-hydroxy-1,1,1-trifluoroethyl)ethylamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fl, fluorescence; Chl, chlorophyll; MES, 4-(morpholinooethanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; MALDI-MS, MALDI mass spectrometry.

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protein, PsbP, and loss of the 17-kDa protein, PsbQ, suggesting that the C-terminal extension in pD1 interferes with the association of these extrinsic proteins (18). Additionally, it has been suggested that the extrinsic PsbO protein binds with lower affinity to PSII in the D1 mutant S345P in *Synechocystis* 6803 in which pD1 is not processed (19).

Recent structural studies also emphasize close spatial relationship of the D1 C terminus, the manganese cluster, and the extrinsic proteins. Based on the 3.7-Å structure PSII crystal structure from *Thermosynechococcus vulcanus*, the C-terminal oxygen in the carboxylate of the mature D1 protein at Ala-344 can be a direct ligand to one of the manganese atoms (5). However, a more recent crystal structure from *Thermosynechococcus elongatus* at a 3.5 Å resolution has placed the D1 C terminus near the Ca$^{2+}$ ion of the manganese-calcium cluster but did not include it as a ligand (6). Furthermore, the D1 C terminus makes numerous contacts with the three extrinsic proteins, PsbO, PsbU, and PsbV (6). However, it is difficult to extrapolate a temporal sequence of events from these structural details.

To investigate the temporal position of D1 processing in the PSII assembly pathway, we have isolated PSII complexes containing only pD1 from a CtpA-deficient strain of *Synechocystis* 6803. We were unable to detect any manganese associated with these PSII complexes, indicating that D1 processing is required for any stable manganese interaction. The extrinsic polypeptides of PSII, PsbO, PsbU, and PsbV were also absent, suggesting that the C-terminal extension of pD1 precludes their binding. Finally, one protein, Psb27, was found to be significantly more abundant in such PSII complexes.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions—** *Synechocystis* 6803 cultures were grown at 30 °C under 30 μmol of photons m$^{-2}$ s$^{-1}$ of white light in TES-buffered BG-11 medium (20) with air bubbling. The HT3 strain of *Synechocystis* 6803, which has a histidine tag at the C-terminal end of the CP47 protein (21) was a generous gift from Prof. T. M. Bricker (Louisiana State University, Baton Rouge, LA). Growth medium for the ΔtctA cells was supplemented with 5 mM glucose, 10 μM 3,3-dichlorophenol-indol-1,1-dimethyle (DCMU), and 3 μg/ml ethrytomycin, whereas growth medium for the ΔtctAΔtctTP cells were grown in BG11 medium supplemented with 5 μM glucose in 80% nitric acid and allowed to digest overnight. The digested membranes were then diluted 1:2 in deionized water for metal analysis.

**Membrane Association of PSII Proteins—** Membranes were suspended in HCMS buffer to 800 μg of Chl/ml. The membranes were centrifuged at 3000 g for 3 min to remove the glass beads and unbroken cells. The resulting supernatant was then centrifuged at 40,000 × g for 20 min to pellet the cell membranes. The membrane samples were resuspended in HCMS buffer to ~800 μg of Chl/ml. The membrane samples were diluted to 10 μg of Chl/ml in 80% nitric acid and allowed to digest overnight. The digested membranes were then diluted 1:2 in deionized water for metal analysis. The manganese/PSII ratio was calculated based on 41 molecules of Chl/PSII (2).

**RESULTS**

**ΔtctAΔtctTP Mutant Construction—** To characterize the PSII complexes containing only the pD1 protein, we generated the double mutant ΔtctAΔtctTP in *Synechocystis* 6803, in which (a) the CP47 protein has a C-terminal histidine tag to facilitate PSII isolation (21), and (b) the tctA gene is deleted resulting in the presence of pD1 in the PSII complex (13). The ΔtctA construct used for this study differs from that described previously (13) to be compatible with the kanamycin-resistant HT3 mutation. The ΔtctA and ΔtctAΔtctTP mutants described in the present work contain a 1.5-kb ethrytomycin resistance marker at the tctA locus (Fig. 1A). Complete segregation of the ΔtctA mutation was confirmed by PCR analysis of the tctA locus (Fig. 1B). The presence of the C-terminal histidine tag at the psbB locus was confirmed by nucleotide sequencing (Fig. 1C).

Because the low fluorescence phenotype has been observed for
other D1 processing protease mutants (12, 16, 24), the fluorescence properties of the ΔctpA and ΔctpAHT3 mutants were also examined to verify segregation. Fig. 1D shows that the ΔctpA and ΔctpAHT3 mutants have negligible Kautsky fluorescence induction curves compared with that of HT3 cells.

**Manganese Content of ΔctpAHT3 Membranes and Isolated PSII**—Mutants containing a defective D1 processing protease have PSII complexes with no oxygen evolution activity specifically because of the absence of an active manganese cluster (12, 13, 25). It was previously reported that membranes from the *Scenedesmus* LF1 mutant had a significant decrease in the manganese content. Therefore, it was concluded that the assembly of a functional manganese cluster was not possible in D1 processing mutants, because only 2 of the 4 manganese atoms involved in oxygen evolution activity could bind to PSII (25). However, it was unclear whether the manganese content of membranes was representative of the manganese bound to PSII complexes.

To resolve the issue of the number of manganese atoms associated with pD1-containing PSII, the manganese content of membranes and isolated PSII samples from HT3 and ΔctpAHT3 cells were measured using atomic absorption spectrometry. Table I shows that the manganese content of ΔctpAHT3 membranes is indeed ~50% that of HT3 membranes on a per chlorophyll basis. However, manganese measurements of isolated PSII complexes revealed a different interpretation of the ability of manganese to bind to pD1-containing PSII complexes. As expected, HT3 PSII complexes had a manganese:PSII of 4, but PSII complexes from the ΔctpAHT3 mutant were devoid of any manganese.

Another widely used technique to examine the presence of a functional manganese cluster in PSII is to measure the decay of chlorophyll fluorescence after a single saturating flash in the presence of DCMU. Upon illumination, charge separation gives rise to a fluorescence decay after a single saturating flash in the presence of DCMU. The decay curve is characteristic of charge recombination between QA and QB electron acceptors within PSII. The presence of DCMU prevents normal QA to QB electron transfer. Thus, chlorophyll fluorescence decay in the presence of DCMU reflects charge recombination between QA and QB (26). However, the addition of exogenous Mn2+ ions can block charge recombination between QA and QB by donating electrons directly to YZ+ (27). The site at which this Mn2+ ions is bound and oxidized is predicted to be the first binding site during manganese cluster assembly, also referred to as the high affinity manganese binding site (9, 27).

Exogenous Mn2+ ions block charge recombination between QA and YZ+ over a timescale of 1 s (27). This property has been previously used to monitor the integrity of the high affinity manganese binding site in various D1 mutants (9, 27, 28). Fig. 2 shows the charge recombination kinetics between QA and the donor side of ΔctpAHT3 PSII in the presence and absence of 1 mM MnCl2. In the absence of added Mn2+, the decay curve is characteristic of charge recombination between QA and YZ+ with a t1/2 of 20 ms. Similar decay curves and t1/2 values (10–20 ms) have been reported for the *Scenedesmus* LF-1 mutant and the *Synechocystis* D1 S345P and A344Stop mutants (9). As evident from the lack of decay after 600 ms, the addition of exogenous Mn2+ ions blocks charge recombination between QA and YZ+ in ΔctpAHT3 PSII. The structure of the high affinity binding site is not significantly altered in the ΔctpAHT3 mutant, because Mn2+ ions could still access and reduce YZ+.

These results suggest that manganese affinity to PSII is severely decreased in the absence of D1 processing. Because the manganese content of membranes from the ΔctpAHT3 mu-
Finally, although the extrinsic protein PsbV could be detected in HT3 membranes on a per chlorophyll basis. Consistently less (55%) than that in HT3 membranes on a per membrane component.

The presence of PSII proteins in intact thylakoid membranes was also measured to determine whether these proteins were stably associated with the membrane. These data from isolated PSII complexes suggest that D1 cleavage is a requirement for the stable interaction of any of the four manganese atoms with PSII.

**ΔctpA**Δ**HT3 PSII Complexes Lack the Extrinsic Proteins PsbO, PsbV, and PsbU—It has been reported previously that pD1 is incorporated into PSII complexes containing the membrane components D2, CP47, CP43, and cytochrome b_{550} (12, 13, 18, 29). Metz et al. (18) also observed a decrease in the affinity of the extrinsic PsbP and PsbQ proteins for the thylakoid membranes in the *Scenedesmus* LF-1 mutant, suggesting that the presence of the C-terminal extension on D1 interferes with the binding of these components. The ΔctpA**HT3 mutant allowed a more detailed characterization of the protein components of pD1-containing PSII complexes.

The polypeptide profiles of PSII isolated from HT3 and ΔctpA**HT3 strains are shown in Fig. 3. The proteins were identified based on their migration in the SDS-PAGE system described in Ref. 2 or by immunodetection. As expected, ΔctpA**HT3 PSII complexes contained only pD1 and no processed D1. Notably, among the extrinsic proteins, PsbO, PsbQ, and PsbV could not be detected on immunoblots of ΔctpA**HT3 PSII (data not shown). Although the PsbQ protein has not been identified in PSII structural studies, it has recently been identified as a regulatory component of cyanobacterial PSII (30). PsbU could not be detected on Coomassie-stained gels of the mutant PSII sample.

The presence of PSII proteins in intact thylakoid membranes was also measured to determine whether these proteins were in fact expressed in the ΔctpA**HT3 mutant. HT3 and ΔctpA**HT3 cells were broken with glass beads to obtain intact right-side-out thylakoid membranes (31) and probed for the PSII intrinsic and extrinsic proteins as shown in Fig. 4. The intrinsic PSII components, CP47, D2, D1/pD1, and the extrinsic protein PsbO were present in membranes from both HT3 and ΔctpA**HT3. Finally, although the extrinsic protein PsbV could be detected by heme staining in ΔctpA**HT3 membranes, the amount of it was consistently less (55%) than that in HT3 membranes on a per chlorophyll basis.

The results described above indicate that the extrinsic proteins are expressed and properly localized to the thylakoid lumen but do not bind to PSII in ΔctpA**HT3 cells. To further test this conclusion, membranes from HT3 and ΔctpA**HT3 cells were treated with 0.04% β-dodecyl maltoside to partially solubilize the membranes. Detergent-treated samples were centrifuged to separate solubilized proteins from membrane-associated proteins. Fig. 5 shows that the membrane protein, D2, partitions exclusively in the membranes fraction in both HT3 and ΔctpA**HT3 samples. This control ensures that the detergent treatment did not extract membrane proteins. Notably, the HT3 and ΔctpA**HT3 samples differ in the distributions of the extrinsic proteins PsbO and PsbV (Fig. 5). In the HT3 samples, the PsbO (90%) and PsbV (94%) proteins predominantly partition with the membrane fraction indicating that they are still associated with the membrane because of their interactions with the integral membrane protein components of PSII. In the ΔctpA**HT3 samples, more of the PsbO (50%) and PsbV (63%) proteins partition into the soluble fraction suggesting a decreased affinity of these proteins for the PSII integral membrane components. Thus, although the extrinsic proteins are localized to the thylakoid lumen, they are not attached to
DISCUSSION

D1 Processing and Manganese Ion Binding—It has been previously established that D1 processing mutants do not evolve oxygen, because these mutants cannot completely assemble the tetra-nuclear manganese cluster, the catalytic core of the oxygen-evolving complex (9, 12, 13, 16, 25). However, it has been difficult to accurately measure the amount of manganese bound to PSII complexes that contain only pD1. The manganese content of thylakoid membranes from the LF1 mutant of *Scenedesmus* was approximately one-third that of wild type thylakoid membranes. Based on these measurements, it was concluded that only 1–2 manganese atoms can associate with PSII in the absence of D1 processing (16, 25). In an EPR study, Rutherford *et al.* (17) showed that the LF1 mutant lacked the S₂ multiline signal normally attributed to the multivalent manganese cluster. Our data on *Synechocystis* agree with these previous reports. At the level of membranes, the ΔcfpAHT3 mutant has approximately half of the manganese content of HT3. However, our data showed that this is not indicative of manganese binding to PSII, because purified PSI complexes from the ΔcfpAHT3 mutant do not contain any manganese atoms. We concluded that the presence of the D1 C-terminal extension prevents stable association of any manganese atom to PSII.

These results also provide some insights into the nature of the high affinity manganese binding site. A large body of evidence indicates Asp-170 in the D1 protein forms part of the binding site for the first manganese atom bound during manganese cluster assembly (33, 34). This high affinity binding site can be monitored by the ability of exogenous Mn²⁺ ions to reduce Yz⁺ and block charge recombination between Yz⁺ and Qₐ⁻ (9, 27). Site-directed mutants of Asp-170 require much higher Mn²⁺ concentrations to reduce Yz⁺ compared with wild type (27). Nixon *et al.* (9) have reported that although the D1 mutants S345P and A344Stop in *Synechocystis* 6803 cannot assemble a functional manganese cluster, these mutants do retain an intact high affinity site using the assay described above. We showed that the high affinity site of ΔcfpAHT3 PSII is still accessible to exogenous Mn²⁺ ions as measured by fluorescence decay kinetics. However, our atomic absorption data indicate that there is no manganese bound to PSII from this mutant. Thus, although the high affinity site is not perturbed in ΔcfpAHT3, it is not occupied by any manganese atom after purification of PSII.

The absence of manganese atoms in pD1-containing PSII complexes indicates that the C terminus of the mature D1 protein at Ala-344 is crucial to manganese cluster assembly. Indeed, the carboxylate group of Ala-344 was suggested to be a ligand to one of the manganese ions in two different studies (5, 35). However, in the most recent crystal structure of PSII (6), the D1 C terminus is closest to the Ca²⁺ ion of the manganese-calcium cluster. The Ca²⁺ ion is required for the assembly of the manganese cluster (36), and it is possible that the perturbation of its binding site would also result in a failure to assemble the complete manganese cluster. Another possibility, to reconcile all of these data, is that the ligands to the manganese cluster during PSII assembly are different from those of the functional manganese cluster during the water-oxidation reaction.

D1 Processing and Extrinsic Protein Association—Although there has been much scrutiny of the effects of the D1 C terminus on the manganese cluster, relatively little has been known about how the C-terminal extension affects the binding of the extrinsic proteins associated with the oxygen-evolving complex on the luminal side of PSII. Analysis of the LF1 mutant of *Synechocystis* showed reduced binding of the PsbP protein and loss of the PsbQ protein when the thylakoid membranes were treated such that the luminal side was exposed to the buffer (18). This earlier result suggests that the D1 C-terminal extension interferes with the binding of these extrinsic components. However, no further analysis with purified PSI particles from a D1 processing mutant was conducted. Based on the similarity of charge recombination kinetics in the S345P and S345P/ΔpsbO mutants, Chu *et al.* (19) have also postulated that the PsbO protein binds with lower affinity to PSII in the D1 mutant S345P in *Synechocystis* 6803.

Our results demonstrated that the extrinsic proteins PsbO, PsbU, and PsbV are expressed and properly localized but do not associate with PSII complexes containing only pD1. Notably, in the most recent PSI crystal structure the extrinsic proteins PsbO, PsbU, and PsbV have some direct interactions with the luminal C-terminal portion of the mature D1 polypeptide (6). It is possible that when the C-terminal extension is present, it disrupts the structure of the entire luminal C-terminal portion of the D1 polypeptide resulting in an absence of binding of the extrinsic subunits. It has also been reported that the affinity of the extrinsic proteins is substantially decreased in the absence of the manganese cluster (37, 38). Thus, the absence of manganese in ΔcfpAHT3 PSI complexes could also contribute to the loss of the extrinsic proteins.
Possible Role of Psb27 in PSII Assembly—The Psb27 (Srl1645) protein was found to be more abundant in PSII complexes from the ΔctpaH3T mutant as compared with PSII complexes isolated from wild type cells. Psb27 is an 11-kDa polypeptide first identified in a PSII preparation from Synechocystis 6803 (32). This protein was originally named PsbZ but according to a new nomenclature has been renamed Psb27 (2). Homologues of Psb27 are found in plants and all cyanobacterial genomes analyzed to date, except Gloeobacter violaceus (40–45). A sequence analysis indicates that Psb27 contains an N-terminal cleavable signal peptide recognized by bacterial signal peptidase II. Thus, Psb27 is predicted to be a luminal-localized extrinsic protein. Indeed, the Arabidopsis homologues (At1g03600 and At1g05385) of Psb27 have been identified in a study of the thylakoid lumen proteome (39).

Despite the fact that Psb27 homologues have been identified in numerous photosynthetic organisms, the function of Psb27 within PSII remains unknown. Furthermore, it has not been observed in any of the recent PSII crystal structures. Because it accumulates in ΔctpaH3T PSII complexes, which are arrested in the PSII assembly process, Psb27 may play a role during PSII biogenesis. It is possible that Psb27 associates transiently with PSII during assembly but is not part of the assembled functional complex. It is noteworthy that PSII complexes isolated from wild type cells represent a population of PSII at all stages of assembly. Therefore, Psb27 in this predicted role would still be expected to associate with a fraction of the PSII complexes. Further studies are necessary to determine the nature of the association of Psb27 with PSII and role of Psb27 in PSII assembly.

Temporal Order of Events during PSII Biogenesis—The ΔctpaH3T mutant provides a means to study PSII complexes during an early stage of the biogenesis pathway. Previously it has been shown that the D1 protein is first synthesized in its precursor form, inserted into the thylakoid membrane, and assembled into a PSII complex capable of electron transfer from Yz to Qa (9, 13, 16, 25). Our results now place the D1 processing and PSII assembly mutants to further define the dynamic nature of PSII assembly.

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