

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

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**Photosystem II (PSII) is a large membrane protein complex that catalyzes oxidation of water to molecular oxygen. During its normal function, PSII is damaged and frequently turned over. The maturation of the D1 protein, a key component in PSII, is a critical step in PSII biogenesis. The precursor form of D1 (pD1) contains a C-terminal extension, which is removed by the protease CtpA to yield PSII complexes with oxygen evolution activity. To determine the temporal position of D1 processing in the PSII assembly pathway, PSII complexes containing only pD1 were isolated from a CtpA-deficient strain of the cyanobacterium *Synechocystis* 6803. Although membranes from the mutant cell had nearly 50% manganese, no manganese was detected in isolated  $\Delta$ ctpAHT3 PSII, indicating a severely decreased manganese affinity. However, chlorophyll fluorescence decay kinetics after a single saturating flash suggested that the donor  $Y_Z$  was accessible to exogenous  $Mn^{2+}$  ions. Furthermore, the extrinsic proteins PsbO, PsbU, and PsbV were not present in PSII isolated from this mutant. However, PsbO and PsbV were present in mutant membranes, but the amount of PsbV protein was consistently less in the mutant membranes compared with the control membranes. We conclude that D1 processing precedes manganese binding and assembly of the extrinsic proteins into PSII. Interestingly, the Psb27 protein was found to be more abundant in  $\Delta$ ctpAHT3 PSII than in HT3 PSII, suggesting a possible role of Psb27 as an assembly factor during PSII biogenesis.**

phytins, plastoquinones, manganese, non-heme iron, calcium, and chloride atoms as well as two heme groups (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 *Scenedesmus obliquus* LF1 mutant suggests that pD1-containing PSII complexes have only 1–2 manganese/PSII (16). EPR studies of the LF1 mutant revealed the lack of the  $S_2$  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

Photosystem II (PSII),<sup>1</sup> 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 including both membrane-integral and extrinsically associated proteins (2, 3). In addition to its protein components, PSII also has many associated cofactors including chlorophylls, pheo-

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<sup>1</sup> The abbreviations used are: PSII, photosystem II; pD1, precursor D1; TES, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F, fluorescence; Chl, chlorophyll; MES, 4-morpholineethanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; MALDI-MS, MALDI mass spectrometry.

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 Pso 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<sup>2+</sup> 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, Pso, PsoU, and PsoV (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 atoms associated with these PSII complexes, indicating that D1 processing is required for any stable manganese interaction. The extrinsic polypeptides of PSII, Pso, PsoU, and PsoV were also absent, suggesting that the C-terminal extension of pD1 precludes their binding. Finally, one protein, Pso27, 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<sup>-2</sup> s<sup>-1</sup> of white light in TES-buffered BG-11 medium (20) with air bubbling. The HT3 strain of *Synechocystis* 6803, which has a hexahistidine 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 *ΔctpA* cells was supplemented with 5 mM glucose, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and 3 μg/ml erythromycin, whereas growth medium for the *ΔctpAHT3* cells was supplemented with 5 mM glucose, 10 μM DCMU, 5 μg/ml kanamycin, and 3 μg/ml erythromycin. Because the *ΔctpA* and *ΔctpAHT3* cells are light-sensitive, they were cultured at a lower light intensity by wrapping culture bottles in Kimwipes.

**Mutant Construction**—The *ΔctpA* deletion construct was prepared as follows. A 6-kb BamHI/HindIII fragment of *Synechocystis* 6803 DNA containing the *ctpA* locus was cloned into a pUC19 vector lacking an EcoRI site (pSL794). The *ctpA* locus within pSL794 (1.4 kb), which is flanked on either side by EcoRI sites, was replaced with a 1.5-kb EcoRI fragment containing an erythromycin resistance marker (pSL795). The double mutant *ΔctpAHT3* was constructed by transforming HT3 cells with the *ΔctpA* construct. Complete segregation of the *ΔctpA* mutation was determined by PCR using the following primers, 5'-AAGTCCAT-GCTGTGGAAGC-3' and 5'-GGATGCCTTACTTATGGC-3'.

**Fluorescence Measurements**—Fluorescence measurements were conducted to test the segregation of the *ΔctpA* mutation by verifying the low fluorescent *ΔctpA* phenotype. Briefly, HT3, *ΔctpA*, and *ΔctpAHT3* cells were grown in BG11 medium supplemented with 5 mM glucose in 12-well microtiter plates. *F*<sub>o</sub> and Kautsky fluorescence induction were measured on a FluorCam 690M (Photon Systems Instruments, Brno, Czech Republic). The average *F*<sub>o</sub> was calculated from a 3-s measurement before actinic illumination. Samples were then subjected to continuous actinic light (300 μmol of photons m<sup>-2</sup> s<sup>-1</sup>) at 30% intensity for 10 s and fluorescence (*F*) was monitored by 33-μs measuring flashes every 40 ms. Fluorescence traces were normalized to *F*<sub>o</sub> to compare the signals from different strains.

Chlorophyll fluorescence decay after a single saturating flash was measured using an FL100 fluorometer (Photon Systems Instruments, Brno, Czech Republic). PSII samples were diluted to 2 μg of Chl/ml in resuspension buffer (50 mM MES-NaOH, pH 6.0, 10 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, and 25% glycerol). Samples were incubated under ambient light

for 10 min with and without the addition of 1 mM MnCl<sub>2</sub> followed by an incubation in darkness for 10 min. DCMU was added to a final concentration of 10 μM just prior to measurement. The average *F*<sub>o</sub> was calculated as the average signal after four measuring flashes from the dark-adapted sample. Samples were subjected to a 30-μs saturating actinic flash to give *F*<sub>m</sub> followed by a series of 3-μs measuring flashes to follow *F* over a 1-s measurement period. The fluorescence signals were normalized as (*F* - *F*<sub>o</sub>)/(*F*<sub>m</sub> - *F*<sub>o</sub>).

**Isolation of PSII**—PSII preparations from the HT3 and *ΔctpAHT3* strains were isolated according to Ref. 2.

**Determination of Manganese Content**—The concentrations of manganese were measured on an AA600 atomic absorption spectrophotometer (PerkinElmer Life Sciences). Membrane samples were prepared as follows. Cells were harvested and washed with 20 mM HEPES, pH 7.8, 5 mM EDTA buffer according to Ref. 22, resuspended in HCMS buffer (50 mM HEPES-NaOH, 5 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, and 1 M sucrose, pH 7.8) to a concentration of 500 μg of Chl/ml, and broken with 0.17-mm glass beads. The cell lysate was 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 membranes 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. PSII samples were diluted to 5 μg of Chl/ml in deionized water. The manganese:PSII ratio was calculated based on 41 molecules of Chl/PSII (2).

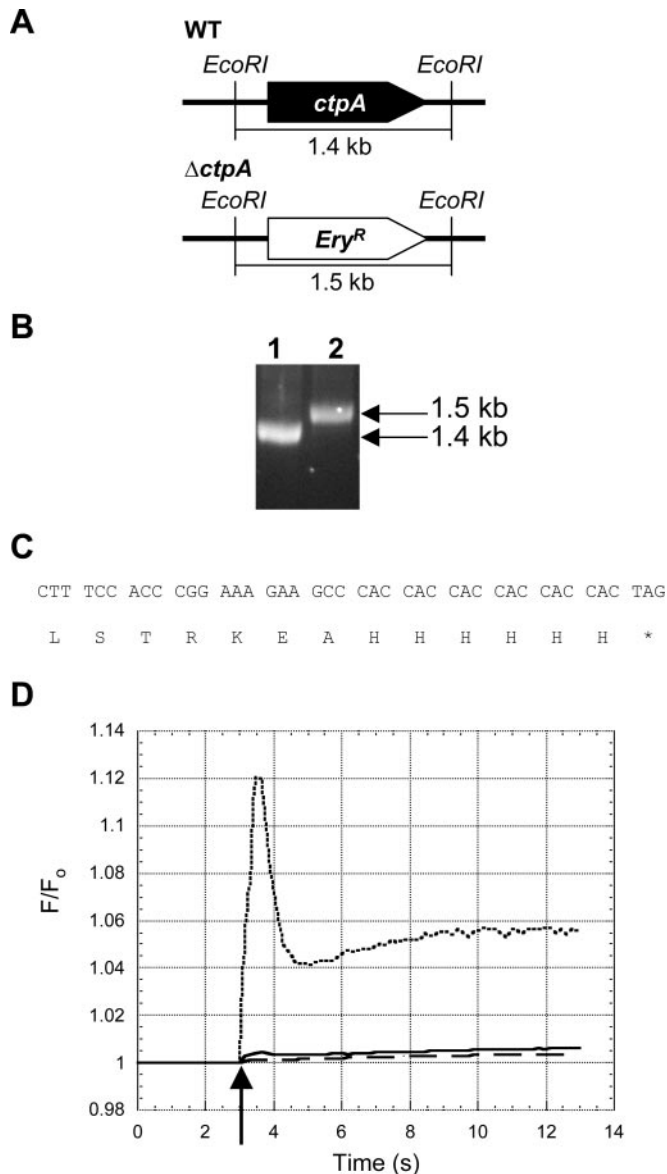
**Protein Detection**—PSII samples (7 μg of Chl/lane) were fractionated using the SDS-PAGE system described in Ref. 2 and visualized with Coomassie Blue staining (Sigma). Membrane samples (5 μg of Chl/lane) were fractionated using a 16% acrylamide, 6 M urea SDS-PAGE system. For immunodetection, fractionated proteins were blotted onto nitrocellulose membranes, and the CP47, D1, D2, and Pso proteins were identified using the corresponding specific antibodies. The hybridization signals were detected using chemiluminescence reagents (Pierce) and then visualized on a Fujifilm LAS-1000 plus imager (Fujifilm, Stamford, CT). PsoV (cytochrome *c*<sub>550</sub>) was detected based on heme staining using chemiluminescence reagents. The identity of the Pso27 band was confirmed by MALDI-MS analysis (see below).

**MALDI Mass Spectrometry**—The protein band corresponding to Pso27 was excised from a Coomassie-stained gel, reduced with 10 mM dithiothreitol for 30 min, alkylated with 55 mM iodoacetamide for 30 min, and digested with trypsin at 37 °C for 12 h. The resulting peptide fragments were analyzed on a MALDI time-of-flight Voyager DE STR instrument (Applied Biosystems, Foster City). The Protein Prospector program was used to identify hits in the *Synechocystis* 6803 translated open reading frames data base (46).

**Membrane Association of PSII Proteins**—Membranes were partially solubilized as described in Ref. 23 with some modifications. Briefly, cells were washed with 20 mM HEPES, pH 7.8, 5 mM EDTA and broken with glass beads. The resulting cell lysate (~300 μg of Chl/ml) was treated with 0.04% (w/v) of the detergent β-dodecyl maltoside (Amresco) and immediately centrifuged at 60,000 × *g* for 40 min to pellet the membranes. The supernatant fraction was carefully removed, and the membranes were suspended in HCMS buffer to 800 μg of Chl/ml. Supernatant and membranes samples were analyzed for PSII protein content. The ImageJ program was used to quantify the relative amounts of different proteins in the samples.

#### RESULTS

***ΔctpAHT3* Mutant Construction**—To characterize the PSII complexes containing only the pD1 protein, we generated the double mutant *ΔctpAHT3* in *Synechocystis* 6803, in which (a) the CP47 protein has a C-terminal histidine tag to facilitate PSII isolation (21), and (b) the *ctpA* gene is deleted resulting in the presence of pD1 in the PSII complex (13). The *ΔctpA* construct used for this study differs from that described previously (13) to be compatible with the kanamycin-resistant HT3 mutation. The *ΔctpA* and *ΔctpAHT3* mutants described in the present work contain a 1.5-kb erythromycin resistance marker at the *ctpA* locus (Fig. 1A). Complete segregation of the *ΔctpA* mutation was confirmed by PCR analysis of the *ctpA* locus (Fig. 1B). The presence of the C-terminal hexahistidine tag at the *psbB* locus was confirmed by nucleotide sequencing (Fig. 1C). Because a low fluorescence phenotype has been observed for



**FIG. 1. Construction of  $\Delta ctpA$ HT3 mutant.** *A*, the *ctpA* locus in wild type and  $\Delta ctpA$  strains of *Synechocystis* 6803. *B*, PCR of *ctpA* locus from wild type cells (1) and  $\Delta ctpA$ HT3 cells (2). *C*, sequence analysis of a segment of the *psbB* locus in the  $\Delta ctpA$ HT3 strain. The *top line* shows the nucleotide sequence, and the *bottom line* shows the deduced amino acid sequence. *D*, measurement of  $F_o$  and Kautsky fluorescence induction for HT3 (dotted),  $\Delta ctpA$  (solid), and  $\Delta ctpA$ HT3 (dashed) cells. Arrow indicates the onset of continuous actinic light. For more details, see “Materials and Methods.”

other D1 processing protease mutants (12, 16, 24), the fluorescence properties of the  $\Delta ctpA$  and  $\Delta ctpA$ HT3 mutants were also examined to verify segregation. Fig. 1D shows that the  $\Delta ctpA$  and  $\Delta ctpA$ HT3 mutants have negligible Kautsky fluorescence induction curves compared with that of HT3 cells.

**Manganese Content of  $\Delta ctpA$ HT3 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 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

TABLE I

## Manganese content of membranes and purified PSII samples

Total membranes from HT3 and  $\Delta ctpA$ HT3 cells were prepared as described under “Materials and Methods” and digested in 80% nitric acid. 5  $\mu$ g of Chl-containing PSII samples were diluted in deionized water. Relative percentages are given in parentheses. Mn:PSII ratios were calculated based on 41 Chl/PSII (2). For more details, see “Materials and Methods.” ND, not determined; Mn, manganese.

	Mn/Chl	Mn/PSII
	nmol/mol	
HT3 membranes	32.3 $\pm$ 1.2 (n = 3) (100%)	ND
$\Delta ctpA$ HT3 membranes	16.3 $\pm$ 2.5 (n = 3) (50.5%)	ND
HT3 PSII	98.0 $\pm$ 4.8 (n = 4) (100%)	4.0 $\pm$ 0.2 (n = 4)
$\Delta ctpA$ HT3 PSII	1.0 $\pm$ 0.1 (n = 4) (1.1%)	0.04 $\pm$ 0.01 (n = 4)

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  $\Delta ctpA$ HT3 cells was measured using atomic absorption spectroscopy. Table I shows that the manganese content of  $\Delta ctpA$ HT3 membranes is indeed  $\sim$ 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  $\Delta ctpA$ HT3 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 the high fluorescent state ( $Y_Z^+ P680 Q_A^-$ ), which decays over time as charge recombination occurs between  $Q_A^-$  and other electron acceptors within PSII. The presence of DCMU prevents normal  $Q_A^-$  to  $Q_B$  electron transfer. Thus, chlorophyll fluorescence decay in the presence of DCMU reflects charge recombination between  $Q_A^-$  and the donor side of PSII. In PSII complexes without a functional manganese cluster, charge recombination occurs between  $Q_A^-$  and  $Y_Z^+$  with a  $t_{1/2} \geq 1$  ms (26). However, the addition of exogenous  $Mn^{2+}$  ions can block charge recombination between  $Q_A^-$  and  $Y_Z^+$  by donating electrons directly to  $Y_Z^+$ . The site at which this  $Mn^{2+}$  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  $Mn^{2+}$  ions block charge recombination between  $Q_A^-$  and  $Y_Z^+$  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  $Q_A^-$  and the donor side of  $\Delta ctpA$ HT3 PSII in the presence and absence of 1 mM  $MnCl_2$ . In the absence of added  $Mn^{2+}$ , the decay curve is characteristic of charge recombination between  $Q_A^-$  and  $Y_Z^+$  with a  $t_{1/2}$  of 20 ms. Similar decay curves and  $t_{1/2}$  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  $Mn^{2+}$  ions blocks charge recombination between  $Q_A^-$  and  $Y_Z^+$  in  $\Delta ctpA$ HT3 PSII. The structure of the high affinity binding site is not significantly altered in the  $\Delta ctpA$ HT3 mutant, because  $Mn^{2+}$  ions could still access and reduce  $Y_Z^+$ .

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  $\Delta ctpA$ HT3 mu-

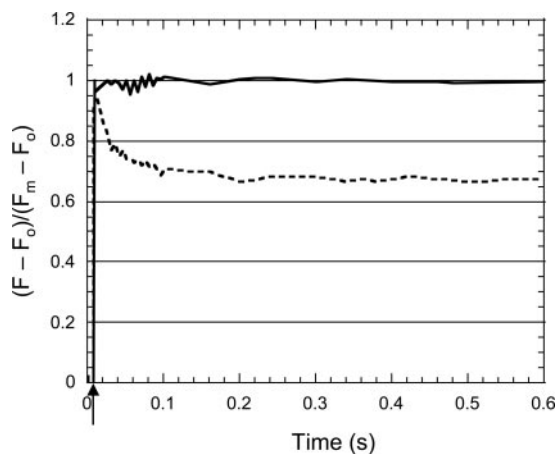


FIG. 2. Charge recombination kinetics between  $Q_A^-$  and the donor side of  $\Delta ctpAHT3$  PSII in the presence and absence of  $Mn^{2+}$ . The charge recombination kinetics of isolated  $\Delta ctpAHT3$  PSII complexes ( $2 \mu\text{g}$  of Chl/ml) were measured in the presence (solid) and absence (dashed) of  $1 \text{ mM MnCl}_2$  as described under "Materials and Methods." The arrow indicates the saturating actinic flash.

tant is  $\sim 50\%$  of that of HT3, it is possible that the PSII-associated manganese does not survive the PSII isolation procedure or that there is another protein(s) that bind manganese in 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.

*$\Delta ctpAHT3$  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  $b_{559}$  (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  $\Delta ctpAHT3$  mutant allowed a more detailed characterization of the protein components of pD1-containing PSII complexes.

The polypeptide profiles of PSII isolated from HT3 and  $\Delta ctpAHT3$  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,  $\Delta ctpAHT3$  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  $\Delta ctpAHT3$  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  $\Delta ctpAHT3$  mutant. HT3 and  $\Delta ctpAHT3$  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  $\Delta ctpAHT3$ . Finally, although the extrinsic protein PsbV could be detected by heme stain in  $\Delta ctpAHT3$  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  $\Delta ctpAHT3$  cells. To further

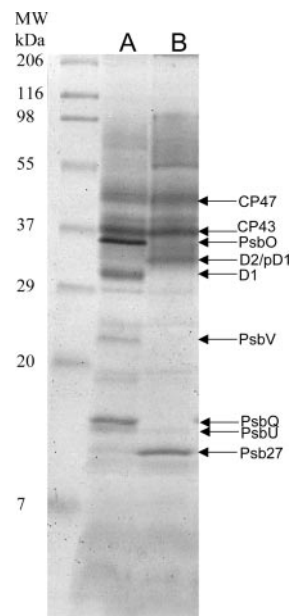


FIG. 3. Polypeptide profiles of isolated PSII. A, HT3 PSII; B,  $\Delta ctpAHT3$  PSII. Selected PSII proteins are shown by arrows on the right. Masses of the molecular weight (MW) standards are indicated on the left.  $7 \mu\text{g}$  of Chl-containing samples were applied to each lane and fractionated by SDS-PAGE, and the polypeptides were stained with Coomassie Blue.

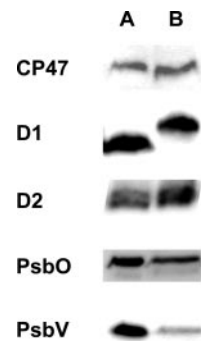


FIG. 4. PSII polypeptides in membranes. A, HT3 membranes; B,  $\Delta ctpAHT3$  membranes.  $5 \mu\text{g}$  of Chl-containing membranes samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed for the indicated PSII proteins. Specific antibodies against CP47, D1, D2, and PsbO were used for immunodetection of the corresponding proteins, respectively. PsbV (cytochrome  $c_{550}$ ) was visualized by heme staining using chemiluminescent reagents.

test this conclusion, membranes from HT3 and  $\Delta ctpAHT3$  cells were treated with  $0.04\%$   $\beta$ -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  $\Delta ctpAHT3$  samples. This control ensures that the detergent treatment did not extract membrane proteins. Notably, the HT3 and  $\Delta ctpAHT3$  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  $\Delta ctpAHT3$  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

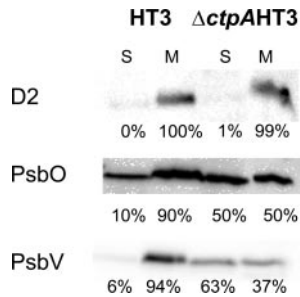


FIG. 5. **Membrane association of PSII proteins.** Membranes isolated from HT3 and  $\Delta$ ctpAHT3 cells were treated with  $\beta$ -dodecyl maltoside as described under "Materials and Methods." The membrane (M) and soluble fractions (S) were analyzed for the presence of the indicated PSII proteins. Antibodies against the D2 and PsbO proteins were used for their immunodetection. PsbV was visualized using heme staining using chemiluminescent reagents. The ImageJ program was used to quantify each band, and the relative amounts are shown.

the mutant PSII complexes and are easily liberated from thylakoid membranes with mild detergent treatment.

**Psb27 Protein Is More Abundant in  $\Delta$ ctpAHT3 PSII Complexes**—During our polypeptide analysis of isolated PSII samples, one unexpected result was the increased abundance of the Psb27 (Slr1645) protein in  $\Delta$ ctpAHT3 PSII relative to HT3 PSII samples (Fig. 3). To confirm that the more abundant protein band on the Coomassie-stained gel was indeed Psb27 and not another co-migrating polypeptide, the band was analyzed by MALDI-MS. The masses of the resulting tryptic fragments accounted for 79% of the mass of the Psb27 protein and none of the masses significantly corresponded to any other *Synechocystis* 6803 protein. Thus, this abundant protein in  $\Delta$ ctpAHT3 PSII is Psb27 and not another polypeptide of similar molecular mass.

The Psb27 protein is  $\sim$ 11-kDa and was originally identified as a component of a purified PSII preparation from *Synechocystis* 6803 based on N-terminal sequencing (32). Sequence analysis indicates that Psb27 is a soluble protein with an N-terminal signal sequence recognized by bacterial signal peptidase II and is predicted to be a lumen-localized extrinsic protein. The function of the Psb27 protein is unknown. The results of this study show the Psb27 polypeptide to be the only luminal extrinsic protein associated with  $\Delta$ ctpAHT3 PSII.

#### 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  $S_2$  multiline signal normally attributed to the multivalent manganese cluster. Our data on *Synechocystis* 6803 agree with these previous reports. At the level of membranes, the  $\Delta$ ctpAHT3 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 PSII complexes from the  $\Delta$ ctpAHT3 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  $Mn^{2+}$  ions to reduce  $Y_Z^+$  and block charge recombination between  $Y_Z^+$  and  $Q_A^-$  (9, 27). Site-directed mutants of Asp-170 require much higher  $Mn^{2+}$  concentrations to reduce  $Y_Z^+$  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  $\Delta$ ctpAHT3 PSII is still accessible to exogenous  $Mn^{2+}$  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  $\Delta$ ctpAHT3, 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  $Ca^{2+}$  ion of the manganese-calcium cluster. The  $Ca^{2+}$  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 *Scenedesmus* 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 PSII particles from a D1 processing mutant was conducted. Based on the similarity of charge recombination kinetics in the S345P and S345P/ $\Delta$ psbO 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 PSII 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  $\Delta$ ctpAHT3 PSII complexes could also contribute to the loss of the extrinsic proteins.

**Possible Role of Psb27 in PSII Assembly**—The Psb27 (Slr1645) protein was found to be more abundant in PSII complexes from the  $\Delta$ ctpAHT3 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 lumen-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  $\Delta$ ctpAHT3 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  $\Delta$ ctpAHT3 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  $Y_Z$  to  $Q_A$  (9, 13, 16, 25). Our results now place the D1 processing event prior to both manganese cluster assembly and extrinsic protein association. Thus, D1 processing is an early event during the formation of the oxygen-evolving complex. Advances in PSII isolation and protein identification techniques will be invaluable tools in the characterization of other PSII assembly mutants to further define the dynamic nature of the PSII complex.

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