Role of the Carboxy Terminus of Polypeptide D1 in the Assembly of a Functional Water-Oxidizing Manganese Cluster in Photosystem II of the Cyanobacterium *Synechocystis* sp. PCC 6803: Assembly Requires a Free Carboxyl Group at C-Terminal Position 344†

Peter J. Nixon,‡ Jeffrey T. Trost, and Bruce A. Diner*

Central Research and Development Department, E. I. Du Pont de Nemours & Company, Experimental Station, P.O. Box 80173, Wilmington, Delaware 19880-0173

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ABSTRACT: The D1 polypeptide of the photosystem II (PSII) reaction center is synthesized as a precursor polypeptide which is posttranslationally processed at the carboxy terminus. It has been shown in spinach that such processing removes nine amino acids, leaving Ala344 as the C-terminal residue [Takahashi, M., Shiraishi, T., & Asada, K. (1988) *FEBS Lett. 240*, 6–8; Takahashi, Y., Nakane, H., Kojima, H., & Satoh, K. (1990) *Plant Cell Physiol. 31*, 273–280]. We show here that processing on the carboxy side of Ala344 also occurs in the cyanobacterium *Synechocystis* 6803, resulting in the removal of 16 amino acids. By constructing a deletion strain of *Synechocystis* 6803 that lacks the three copies of the *psbA* gene encoding D1, we have developed a system for generating *psbA* mutants. Using this system, we have constructed mutants of *Synechocystis* 6803 that are modified in the region of the C-terminus of the D1 polypeptide. Characterization of these mutants has revealed that (1) processing of the D1 polypeptide is blocked when the residue after the cleavage site is changed from serine to proline (mutant Ser345Pro) with the result that the manganese cluster is unable to assemble correctly; (2) the C-terminal extension of 16 amino acid residues can be deleted with little consequence either for insertion of D1 into the thylakoid membrane or for assembly of D1 into a fully active PSII complex; (3) removal of only one more residue (mutant Ala344stop) results in a loss of assembly of the manganese cluster; and (4) the ability of detergent-solubilized PSII core complexes (lacking the manganese cluster) to bind and oxidize exogenous Mn^{2+} by the secondary donor, Z^+, is largely unaffected in the processing mutants (the Ser345Pro mutant of *Synechocystis* 6803 and the LF-1 mutant of *Scenedesmus obliquus*) and the truncation mutant Ala344stop. Our results are consistent with a role for processing in regulating the assembly of the photosynthetic manganese cluster and a role for the free carboxy terminus of the mature D1 polypeptide in the ligation of one or more manganese ions of the cluster.

The D1 polypeptide of the photosystem II (PSII)[1] complex plays a key role in many aspects of PSII function, from the reduction of plastoquinone to plastoquinol on the acceptor side of the complex [reviewed in Diner et al. (1991a)] to the oxidation of water to molecular oxygen on the donor side (Nixon & Diner, 1992). The primary and secondary electron-transfer reactions of PSII occur within the reaction center, a heterodimer of the D1 polypeptide and the related D2 polypeptide (Nanba & Satoh, 1987; Marder et al., 1987), each of which spans the thylakoid membrane five times (Sayre et al., 1986; Trebst, 1986).

The D1 polypeptide of higher plants is unusual among thylakoid membrane proteins in that it is synthesized as a precursor polypeptide of 353 amino acid residues which is processed within 1–2 min of synthesis (Reisfeld et al., 1982; Minami & Watanabe, 1985) by removal of 9 residues from the C-terminus, leaving Ala344 as the terminal residue (Takahashi et al., 1988; Takahashi et al., 1990). Cleavage on the carboxy side of Ala344 also occurs in the green alga *Chlamydomonas reinhardtii* with the removal of eight residues (B. Diner, unpublished data). However, for cyanobacteria there is only indirect evidence to indicate an analogous process (Nixon et al., 1991).

The function, if any, of the extension is unknown. Studies on the LF-1 mutant of the green alga *Scenedesmus obliquus* (Metz et al., 1980), which lacks the protease activity responsible for processing D1 (Taylor et al., 1988a), have linked a failure to cleave the C-terminal extension with an inability to assemble the manganese cluster (Diner et al., 1988; Taylor et al., 1988b). While this interpretation is likely, the possibility that the phenotype observed with LF-1 arises from a block in the processing of some PSII polypeptide other than D1 could not be completely ruled out.

To help understand the relationship between structure and function in the D1 polypeptide and in particular to clarify the role of the C-terminal extension, we describe in this paper the development of a system for the construction of *psbA* mutants in the cyanobacterium *Synechocystis* sp. PCC 6803, an

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* Address correspondence to this author.

‡ Present address: Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, SW7 2AY, England.

[1] Abbreviations: Chl, chlorophyll *a*; cm, chloramphenicol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; kan, kanamycin; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; P_{680}, primary electron donor (a monomer or dimer of chlorophyll a); PCR, polymerase chain reaction; Pheo, pheophytin a (the primary electron acceptor); PSII, photosystem II; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; spec, spectinomycin; ssDNA, single-stranded DNA; Tc, tetracycline; WT, wild type; xxx^R, resistant to antibiotic xxx; xxx^S, sensitive to antibiotic xxx; Z, secondary electron donor (D1-Tyr161).

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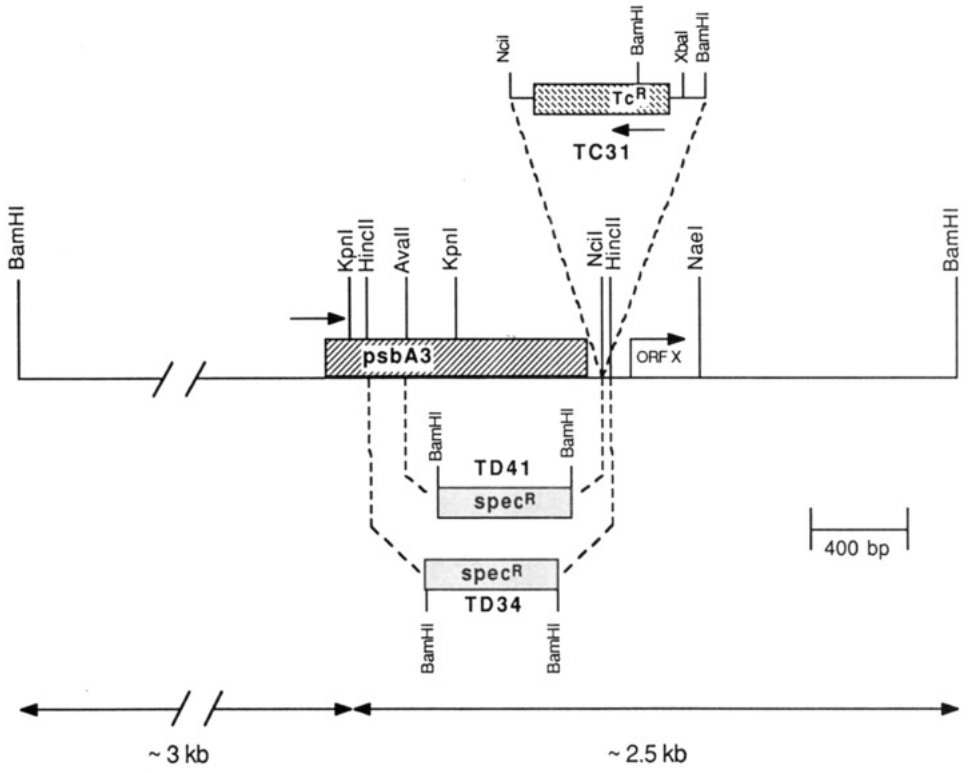

FIGURE 1: Composite map illustrating the mutations made to the *psbA3* gene and flanking regions within the cyanobacterial chromosomes of the deletion strains TD34 and TD41 and the insertion strain TC31. Insertions and deletions within the 5.5-kb *Bam*HI fragment shown are indicated by dashed lines. TD34: deletion of three contiguous *Hinc*II fragments (total of ~1 kb) and replacement by the 2.0-kb spectinomycin-resistance cassette (spec^R) from pHP45Ω. TD41: deletion of the region extending from the *Ava*II site within *psbA3* to the *Nci*I site downstream (~810 bp) and replacement by the same spectinomycin-resistance cassette. TC31: insertion of the ~1.5-kb tetracycline-resistance cassette (Tc^R) into the *Nci*I site downstream of *psbA3*. Only the *Ava*II site referred to in the text and the two *Hinc*II sites that delineate the extent of the deletion in the strain TD34 are indicated. The position and direction of ORF X, downstream of *psbA3*, are indicated by the arrow. The resistance cassettes are not drawn to scale. The extent of the ~2.5 kb of cyanobacterial DNA contained within plasmid pTC3 which was used to construct mutants in the *psbA3* gene is indicated by the right-hand double arrow.

organism widely used for the genetic manipulation of photosynthetic processes (Williams, 1988). The results presented in this paper show that C-terminal processing of D1 occurs in cyanobacteria and that the C-terminal extension is dispensable and does not have an obligatory role in the assembly of a functional PSII complex. Furthermore, we present direct evidence to support the notion that processing of D1 is a prerequisite for assembly of a functional manganese cluster. From the analysis of D1 truncation mutants we also suggest that the C-terminus of the mature D1 polypeptide is involved in assembly of the photosynthetic manganese cluster, possibly through direct ligation to manganese.

MATERIALS AND METHODS

The strain of *Synechocystis* sp. PCC 6803, used for the generation of mutants, is the glucose-tolerant strain originally isolated by J. G. K. Williams (Williams, 1988). This strain was used for the construction of the *psbA* double-deletion strain, $Td_{1,2}$, in which the *psbA1* and *psbA2* genes were replaced by cassettes providing resistance to chloramphenicol (cm) and kanamycin (kan), respectively (Metz et al., 1989). Triple-deletion strain TD34 (Figure 1) was generated from $Td_{1,2}$ by transformation with plasmid pTD3 (Nixon et al., 1990). This plasmid was constructed by the in vitro replacement of three contiguous *Hinc*II fragments, containing all of *psbA3* except for the first 174 bp of the coding region, with a 2.0-kb *Sma*I fragment from plasmid pHP45Ω that encodes resistance to spectinomycin (Prentki & Krisch, 1984).

Conditions for the growth and maintenance of cyanobacterial strains have been described previously (Williams, 1988). When required, glucose (5 mM), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (10 μM), and tetracycline (15 $\mu g \cdot mL^{-1}$) were added to the media at the amounts indicated. DCMU was added to eliminate the influence of active PSII on growth rates and thus forestall reversion. Photoautotrophic growth rates were determined by monitoring the absorbance at 730 nm of 50-mL cultures grown in 125-mL bubbler flasks as described by Williams (1988). Under these conditions, cultures grew exponentially until an absorbance at 730 nm of approximately 1 was reached.

Plasmids pJM10, which contains a 432-bp *Kpn*I fragment internal to the *psbA3* gene (codons 34–179), and pJM13b, which contains a ~2.1-kb *Kpn*I/*Bam*HI fragment of DNA beginning at codon 180 of the *psbA3* gene and ending ~1.6 kb downstream of the stop codon, have been isolated previously (Metz et al., 1989). Plasmid pJM14 is a derivative of pJM13b and contains a spectinomycin-resistance cassette downstream of the *psbA3* gene (Metz et al., 1989). Plasmid pDCTc was used as the source of the tetracycline-resistance cassette and was constructed by subcloning the 1.4-kb *Eco*RI/*Ava*I fragment of pBR322 containing the tetracycline-resistance gene, into the *Hpa*I site of plasmid pDC057, after the ends were made blunt using T4 DNA polymerase. Plasmid pDC057 was made available by Dexter Chisholm and is a derivative of pUC118. Plasmid pTC2, a derivative of Bluescript (BM$^+$ SK), was assembled from the following fragments: a 600-bp *Kpn*I/*Nci*I fragment from pJM13b, encoding residues 180–360 of the *psbA3* gene product together with 53 bp of downstream DNA; a ~1.5-kb *Pst*I fragment from pDCTc that contains the tetracycline-resistance gene from pBR322,

together with some polylinker region; and a ~1.5-kb NciI/XbaI fragment from pJM13b containing DNA from downstream of *psbA3*. Incompatible ends were made blunt using T4 DNA polymerase. Plasmid pTC3 was generated by ligating the 432-bp *Kpn*I fragment of pJM10 into the unique *Kpn*I site of pTC2 to assemble the *psbA3* gene from codons 34 to 360. The *psbA3* gene in pTC3 was sequenced to confirm that no base changes had occurred in the cloning steps. Standard techniques were used to clone and manipulate DNA (Maniatis et al., 1982).

The *psbA*-deletion strain TD41 (Figure 1) was generated by transforming the double-deletion strain $Td_{1,2}$ with plasmid pTD4 and selecting for spectinomycin-resistant colonies grown in the presence of the PSII inhibitor DCMU and glucose (5 mM). Plasmid pTD4 is a Bluescript derivative and contains a ~230-bp *Kpn*I/*Ava*II fragment from pJM10, encoding residues 34–110 of the *psbA3* gene product, in tandem with the 2.0-kb *Sma*I fragment from pHP45Ω that encodes resistance to spectinomycin (Prentki & Krisch, 1984) and ~1.5-kb of DNA from downstream of *psbA3*, from the *Nci*I site through to the *Bam*HI site, which was obtained from pJM13b.

Oligonucleotide-mediated mutagenesis of the *psbA3* gene was accomplished using a Mutagene kit (Bio-Rad Laboratories, Richmond, CA) that uses the enrichment protocol developed by Kunkel et al. (1987). Plasmid pTC3 was transformed into the *dut*⁻ *ung*⁻ host CJ236, and ssDNA was obtained for mutagenesis by superinfecting with phage M13KO7 according to the manufacturer's instructions. Mutagenesis was performed using oligonucleotides of typically 27 bases that had been synthesized on an Applied Biosystems Inc. Model 394 oligonucleotide synthesizer. Usually, the generation or abolition of a restriction endonuclease site at, or close to, the codon to be altered was used as an aid in the identification of mutant plasmids, which were obtained after transformation of *Escherichia coli* (DH5α) and selection on ampicillin (100 $\mu g \cdot mL^{-1}$) and tetracycline (15 $\mu g \cdot mL^{-1}$). Plasmids were sequenced to confirm that the correct alterations in nucleotide sequence had occurred. TD34 or TD41 were transformed with the mutant plasmid according to standard protocols (Williams, 1988). Typically 0.5 mL of cells at an OD_{730nm} of ~2.5 was incubated in the light for ~6 h at 30 °C with DNA at a final concentration of ~40 $\mu g \cdot mL^{-1}$. A total of 0.2 mL of the transformation mix was then plated onto a nitrocellulose filter lying on a glucose plate and incubated for a further 2 days. Two filters were obtained for each transformation: one was transferred to a BG-11 plate to test for restoration of photoautotrophic growth and the other to a glucose plate containing tetracycline (15 $\mu g \cdot mL^{-1}$) and DCMU (10 μM) to select for transformants. The tetracycline plates were incubated under moderate light conditions (~2 W/m^2) for 7–10 days until colonies became visible. As tetracycline is light-sensitive, the filters were usually transferred after 3–4 days to fresh tetracycline plates to maintain selection. Only Tc^R colonies that were sensitive to spectinomycin (50 $\mu g \cdot mL^{-1}$) were analyzed further. This selection ensured that transformants were completely segregated and had not incorporated the Tc^R gene into the chromosome through a single-cross-over recombination event. All mutants were analyzed by Southern blot to confirm that the *psbA3* gene had been returned to the chromosome. Typically, a *Bam*HI digest of genomic DNA was performed, with the presence of a single-hybridizing band of ~5.1 kb, diagnostic for correct incorporation into the chromosome (see Figure 1). The presence or absence of a restriction-site associated with the mutation was also confirmed by Southern analysis.

The presence of the mutation within the chromosome was confirmed by amplifying the *psbA3* gene from the chromosome by asymmetric PCR (McCabe, 1990) using primer ratios of 50:1, removing excess primers with a Centricon 100 microconcentrator (Amicon, Danvers, MA 01923), and sequencing the ssDNA using custom-made oligonucleotides and Sequenase according to the manufacturer's instructions (U.S. Biochemical Corp., Cleveland, OH). As a further check that the mutant phenotype was indeed caused by the site-directed mutation, the mutant strains were restored to photoautotrophy by plasmid DNA containing various portions of the *psbA3* gene. Usually the mutants were transformed by pTC3 (to confirm that the mutation was in *psbA3*), pJM10 (for mutations between residues 34 and 180), pJM14 (for mutations between residues 180 and 360), and the mutant plasmid itself (a negative control).

The C-terminal residue of the mature D1 polypeptide from *Synechocystis* PCC 6803 was identified using the method described by Takahashi et al. (1988) for the determination of the C-terminal residue of D1 from spinach. The starting material was detergent-solubilized PSII core complexes isolated from the phycocyanin-deficient "olive" mutant as described by Rögner et al. (1990) using anion-exchange and hydroxyapatite HPLC. PSII complexes containing approximately 1 mg of chlorophyll were digested with 150 μg of trypsin (Boehringer Mannheim, P.O. Box 50414, Indianapolis, IN 46250) in darkness with gentle agitation for 7 h at 37 °C in a buffer containing 100 mM Tris pH 8.0 and 2 M urea. Peptides of less than 100 kDa were recovered from the digestion using a Centricon 100 microconcentrator. Peptides in the filtrate were separated by reverse-phase HPLC (Hewlett Packard 1090M liquid chromatograph) with a Vydak C_4 column (4.6 × 250 mm, 5-μm beads, 300-Å pore size; The Nest Group, Southborough, MA 01772) using a 0–60% acetonitrile concentration gradient in water containing 0.1% trifluoroacetic acid at a flow rate of 1 $mL \cdot min^{-1}$. Peptides eluting between 22% and 24% acetonitrile were pooled, lyophilized, redissolved in 0.8 mL of 50 mM sodium acetate, pH 5.0, 20 mM $CaCl_2$, applied to an affinity column (1 mL) of anhydrotrypsin-conjugated agarose (Takara Biomedicals, Kyoto, Japan), and washed with 1 column volume of the same buffer. The eluted peptides were then rechromatographed by reverse-phase HPLC as described above. N-Terminal sequence analysis of the tryptic peptides was performed by automated Edman degradation as described by Trower et al. (1990). Fast atom bombardment mass spectroscopy of the C-terminal fragment was performed on a double-focusing mass spectrometer (VG Co. Model ZAB-E) equipped with a xenon gun.

Immunoblotting was performed as described earlier (Nixon et al., 1989) except that protein was separated by SDS–PAGE on 12% (w/v) polyacrylamide gels. Antiserum specific for the D1 polypeptide of spinach was a gift of Dr. Wim Vermaas and was used at a dilution of 1 in 2000. Antiserum raised against the C-terminal extension of the D1 polypeptide of *Synechocystis* 6803 was obtained through Multiple Peptide Systems (San Diego, CA) by immunizing rabbits to a 16-mer oligopeptide (SGEQAPVALTAPAVNG) linked, using glutaraldehyde, to keyhole limpet hemocyanin. The antiserum was affinity purified using the 16-mer linked to a solid support.

Non-oxygen-evolving PSII core complexes lacking Q_B and the Mn cluster were isolated from *Synechocystis* sp. PCC 6803 using the method of Rögner et al. (1990) and from *S.*

obliquus using the method described by Diner et al. (1988), but leaving out the pH 11 treatment of the membrane fragments.

The rates of relaxation of the chlorophyll fluorescence yield following actinic flashes were measured in whole cells using a flash detection spectrophotometer as described in Nixon and Diner (1992). The cells were suspended in BG-11 medium at an OD_{730nm} of 0.9. Unlike in Nixon and Diner (1992), the cells were not pretreated with benzoquinone and ferricyanide.

The measurement of the efficacy of reduction of Z^+ by exogenous Mn^{2+} was performed by following, as a function of $[Mn^{2+}]$, the blockage of $Z^+Q_A^-$ charge recombination at 325 nm following a single saturating flash excitation of non-oxygen-evolving PSII core complexes (Nixon & Diner, 1992; Diner & Nixon, 1992).

The relative PSII reaction center concentration per cell (OD_{730nm} of 0.9) was determined in whole cells according to Nixon and Diner (1992), by measuring the variable chlorophyll fluorescence in the presence of NH_2OH plus DCMU following pretreatment of the cells with benzoquinone and ferricyanide.

Light-saturated rates of oxygen evolution were determined using whole cells suspended in BG-11 medium plus 5 mM glucose containing 0.3 mM 2,6-dichlorobenzoquinone and 1 mM $K_3Fe(CN)_6$ as described in Nixon and Diner (1992). The light-saturated rate typically measured for wild-type *Synechocystis* 6803 was 150–200 μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}.

RESULTS

The D1 Polypeptide Is Cleaved after Ala344 in the Cyanobacterium Synechocystis PCC 6803. The identification of the processing site on the carboxy side of Ala344 in the C-terminal region of the D1 polypeptide has only been determined for the higher plant, spinach (Takahashi et al., 1988, 1990), and the green alga *C. reinhardtii* (B. A. Diner, unpublished data). To determine whether and, if so, where processing occurs in the cyanobacterium *Synechocystis* PCC 6803, we sequenced the C-terminal fragment of D1 obtained after tryptic cleavage of PSII core complexes. Such cleavage of D1 should give the peptide fragment, NAHNFPLDLA, derived from D1 if Ala344 is the C-terminal residue in mature D1. This peptide was synthesized and found to be eluted from the reverse-phase HPLC column at approximately 23% acetonitrile in water (v/v) under the elution conditions used (see Materials and Methods). Following tryptic digestion of PSII core complexes, the proteolytic fragments eluting at 22–24% acetonitrile in water (v/v) were collected and further enriched by adsorption and elution from an anhydrotrypsin–agarose column, which binds all peptides that have an arginine or lysine residue at the C-terminus (Kumazaki et al., 1987). This treatment resulted in a significant depletion in the number and quantity of peptides to be resolved by reverse-phase HPLC. After another purification step on reverse-phase HPLC, two major peptides were obtained. Amino acid sequencing using automated Edman degradation indicated that one of these corresponded to sequence NAHNFPLDLA. Fast atom bombardment mass spectrometry of this sample indicated the presence of two species of size (m/e) 1111.78 and 1149.71, close to the calculated (m/e) value for peptide NAHNFPLDLA of 1111.55 (M + H) and 1149.51 (M + K), respectively (Figure 2). We therefore conclude from these experiments that the translated D1 polypeptide is processed in *Synechocystis* 6803 by the removal of 16 amino acid residues from the C-terminus and that, as in eukaryotes, Ala344 is the C-terminal residue of the mature form of D1.

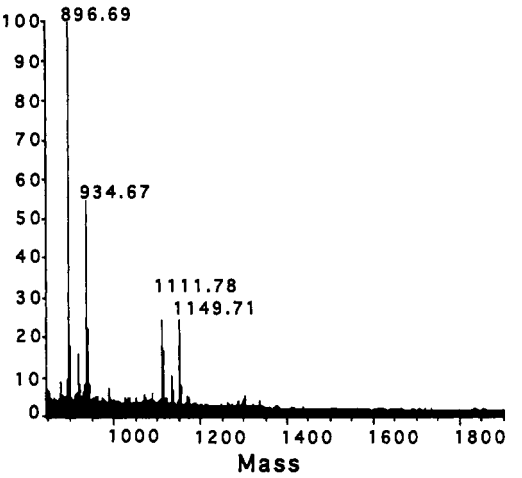

FIGURE 2: Fast atom bombardment mass spectrometry of a C-terminally derived polypeptide following purification by reverse-phase HPLC and passage through an anhydrotrypsin column (see Materials and Methods and Results). The peptide was ionized using a xenon gun. Two species of size 1111.78 and 1149.71 (m/e) are obtained, close to the calculated (m/e) value for peptide NAHNFPLDLA of 1111.55 (M + H) and 1149.51 (M + K), respectively.

Mutagenesis of the D1 Polypeptide of Synechocystis PCC 6803. The D1 polypeptide of *Synechocystis* 6803 is encoded by three different copies of the *psbA* gene termed *psbA1*, *psbA2*, and *psbA3* (Jansson et al., 1987). The *psbA2* and *psbA3* gene code for identical gene products (Ravnikar et al., 1989; Metz et al., 1990) and appear to be the only members expressed under laboratory growth conditions (Mohamed & Jansson, 1989). Our strategy for creating strains of *Synechocystis* 6803 with a modified D1 polypeptide involves (i) creating a deletion strain of *Synechocystis* 6803 that lacks all three copies of the *psbA* gene, (ii) generating an altered *psbA3* gene in vitro through oligonucleotide-mediated mutagenesis, and (iii) restoring the mutant *psbA3* gene to the chromosome of the *psbA*-deletion strain, using a plasmid carrying the mutant gene. Because plasmids carrying the Col E1 replicon fail to replicate in *Synechocystis* 6803, transformants can be selected that have undergone a double-cross-over recombination event so that the mutant *psbA3* gene is now present in the chromosome (Williams, 1988).

Mutagenesis and Restoration of psbA3 to the psbA Triple-Deletion Strains TD34 and TD41. Plasmid pTC3 was constructed (see Materials and Methods) to facilitate the in vitro mutagenesis of the *psbA3* gene (between nucleotides 99 and 1080 of the coding region) and its subsequent restoration to the chromosome of a deletion strain. Because pTC3 is a derivative of the Bluescript series of plasmid vectors, ssDNA can readily be obtained for oligonucleotide-directed mutagenesis using the protocol devised by Kunkel et al. (1987). This plasmid also contains a "fourth" antibiotic resistance marker, that of the tetracycline resistance gene from pBR322, inserted downstream of the *psbA3* gene. This acts as a marker for the mutant *psbA3* gene when it is returned to the deletion strains TD34 or TD41.

In test transformations, TD41 and TD34 were transformed with pTC3 using standard protocols and plated onto glucose plates for 2 days, then transferred to either BG-11 plates (to test for restoration of photosynthetic growth) or plates containing tetracycline (Tc) (at 15 μg·mL^{-1}), DCMU (at 10 μM), and glucose (at 5 mM). These latter conditions were adopted to select for Tc^R colonies under photoheterotrophic conditions, in the absence of a selectional pressure for an active PSII complex. In both cases, pTC3 could restore photoau-

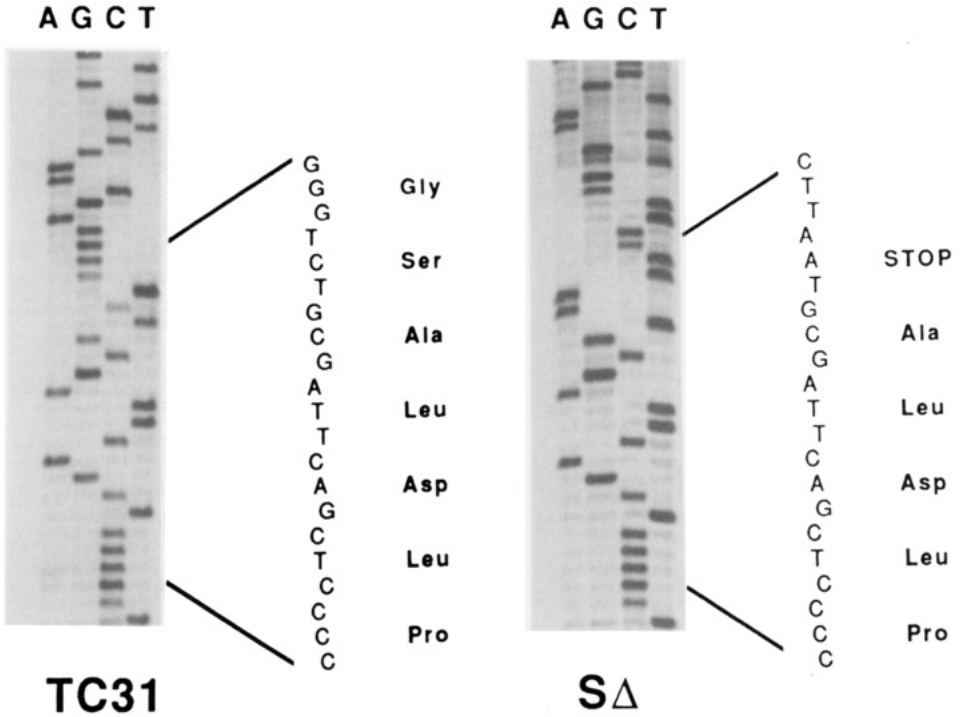

FIGURE 3: Sequencing gel autoradiogram confirming the deletion of the 16 codons encoding the C-terminal extension of D1 in the *psbA3* gene of strain SΔ. Single-stranded DNA containing the *psbA3* gene was amplified from the genomic DNA of strains TC31 and SΔ using asymmetric PCR and sequenced using Sequenase as described in Materials and Methods. For each strain, the DNA sequence of the sense strand (from codons 340 to 346 inclusive) is shown together with the decoded amino acid sequence.

totrophic growth to the deletion strains. For TD41 the frequency of transformation was approximately 10^{-3} μg^{-1} of DNA per viable cell, some 50–100-fold greater than for TD34, presumably because, in one of the two crossover regions, the overlap between the cloned DNA in pTC3 and the DNA left in the chromosome is 75 bp with TD34 and 240 bp with TD41.

A Tc^R transformant, designated TC31, obtained from transformation of TD41, was picked from the Tc, DCMU, glucose plate for further analysis. TC31 was cm^R and kan^R but $spec^S$ indicating that the *psbA3* gene present in plasmid pTC3 had recombined into the chromosome displacing the $spec^R$ cartridge. DNA gel blot analysis (not shown) of genomic DNA obtained from TC31 following digestion with *Bam*HI indicated that the *psbA3* gene was now present on a ~5.1-kb *Bam*HI fragment that also contained much of the Tc^R gene, rather than the ~3-kb fragment found in TD41 (Figure 1). These observations are consistent with incorporation of the *psbA3* gene and Tc^R gene into the chromosome via a double-recombination event. Furthermore, neither the $spec^R$ cassette nor the ~3-kb fragment seen in TD41 could be detected, consistent with complete segregation among the ~10 genome copies in *Synechocystis* 6803 (Labarre et al., 1989).

The fidelity of the recombination of *psbA3* back into the chromosome of the two deletion strains was also examined. Tc^R $spec^S$ colonies isolated under photoheterotrophic growth conditions were tested for photoautotrophic growth on BG-11 plates. Of the 31 colonies tested using TD41 as the host, all were PS^+, indicating accurate recombination. However, when TD34 was used as the host, 8 out of the 12 transformants that were analyzed had undergone illegitimate recombination events either within the $spec^R$ cassette or upstream of the *psbA3* gene (data not shown). TD34 is therefore an unsuitable host for generating *psbA* mutants that are PS^-, although it can be used to assay the ability of mutant plasmids to restore TD34 to photoautotrophic growth. TD41 was therefore used as the host for constructing nonphotosynthetic mutant strains. Consequently for mutagenesis between residues 34 and 110 of D1, using the plasmid pTC3, a proportion of the Tc^R colonies obtained following transformation of TD41 were wild type depending on where recombination had occurred with respect to the site of mutation in the mutant plasmid. For mutations between residues 111 and 360, however, all Tc^R transformants carried the mutation.

The growth rates of TC31 in BG-11 medium (doubling time ~20 h) and in BG-11 medium supplemented by 5 mM glucose (doubling time ~12 h) are indistinguishable from those of the wild type (data not shown). Light-saturated rates of oxygen evolution from whole cells are also similar with rates of approximately 150–200 μmol of O_2·(mg of Chl^{-1})·h^{-1} in BG-11 medium containing 5 mM HCO_3^- in the absence of exogenous acceptors. These results indicate that the presence and orientation of the tetracycline resistance gene in TC31 has little effect on the expression of the *psbA3* gene. TC31 is used as the reference "wild-type" strain for all transformants constructed from TD41 using pTC3.

The C-Terminal Extension Is Not Required for Assembly of D1 into a Functional PSII Complex. The function of the C-terminal extension of D1 is uncertain. One possibility is that it is required for integration of D1 into the thylakoid membrane and another is that it is needed for the accurate assembly of a PSII reaction center and oxygen-evolving complex. To test these possibilities, we used the mutagenesis system described above to construct a deletion strain of *Synechocystis* 6803, designated SΔ, that lacked the 48 bp of the *psbA3* gene encoding the C-terminal extension. Figure 3 shows an autoradiogram of a sequencing gel confirming that the correct deletion has been engineered in strain SΔ and showing the complete absence of WT sequence. Interestingly, SΔ grows photoautotrophically (doubling time of ~20 h) and evolves oxygen (~200 μmol of O_2·(mg of Chl^{-1})·h^{-1}) at wild-type rates. A mutant in which Ser345 was replaced by a stop codon was also constructed. This mutant is indistinguishable from SΔ and behaves like the wild type. We therefore conclude

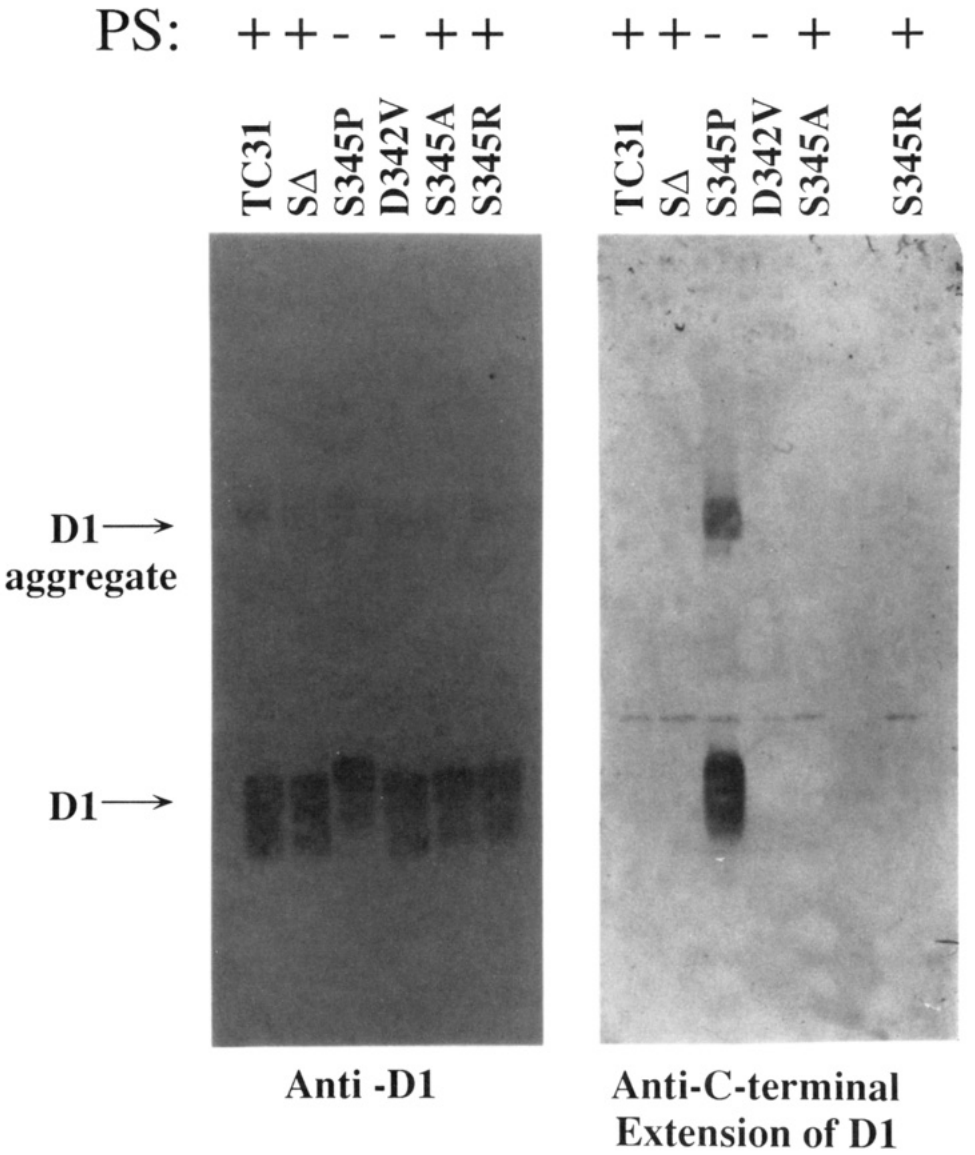

FIGURE 4: Immunoblots of the PSII core complexes isolated from strains TC31, SΔ, Ser345Pro (S345P), Asp342Val (D342V), Ser345Ala (S345A), and Ser345Arg (S345R). Duplicate blots were probed. The left-hand panel was probed with an antibody directed against purified polypeptide D1 (Vermaas et al., 1988). The right-hand panel was probed with an antibody directed specifically against a 16-mer oligopeptide (SGEQAPVALTAPAVNG) corresponding to the C-terminal extension of D1 that is processed in *Synechocystis* 6803 (see Materials and Methods).

that the C-terminal extension is dispensable and is not required for assembly of a functional PSII complex.

Blockage of Processing in Mutant Ser345Pro Prevents Assembly of a Functional Manganese Cluster. The effect on processing of mutating the residue *after* the cleavage site, at Ser-345, was examined by constructing three mutants in which Ser-345 was changed to Ala, Arg, and Pro. Mutants Ser345Ala and Ser345Arg grow photoautotrophically and evolve oxygen at wild-type rates. Judging from the identical electrophoretic mobility of D1 in the mutants and TC31, and the lack of cross-reactivity with an antibody directed against the C-terminal extension, both mutants appear to process the D1 polypeptide (Figure 4). In contrast, mutant Ser345Pro is neither able to grow photoautotrophically nor able to evolve oxygen. While the PSII reaction center levels per cell in this strain are only 20–25% of that in TC31, the absence of any oxygen evolution indicates that those centers that are present do not contain a functional OEC. The D1 polypeptide in this strain also migrates more slowly than in strain TC31 on SDS–PAGE and shows cross-reactivity with the antibody directed against the C-terminal extension of D1 (Figure 4), consistent with a lack of processing of part or all of the C-terminal extension.

To define more precisely the nature of the disruption in electron transfer within the PSII complex of the Ser345Pro mutant, we measured the relaxation of the relative quantum yield of variable chlorophyll fluorescence using a train of weak probe flashes given after each of five saturating actinic flashes, spaced 0.6 s apart (Figure 5) as described by Nixon and Diner (1992). The fluorescence yield is a sensitive measure of the oxidation state of redox components associated with the PSII reaction center. The state $P_{680}PheoQ_A$ gives a low quantum yield of fluorescence (F_0, typically 3% in green plants and algae) and that of $P_{680}PheoQ_A^-$, the highest fluorescence yield (F_{max}, typically 10%). The dependence of the fluorescence yield on $[Q_A^-]$ is nonlinear where energy transfer occurs between centers but linear in the absence of energy transfer (Joliot & Joliot, 1964). The former situation applies in *Scenedesmus* while the latter situation appears to prevail in *Synechocystis* 6803.

The charge-separated state $ZP_{680}^+PheoQ_A^-Q_B$, generated within 300 ps of excitation of P_{680}, is in a low fluorescence

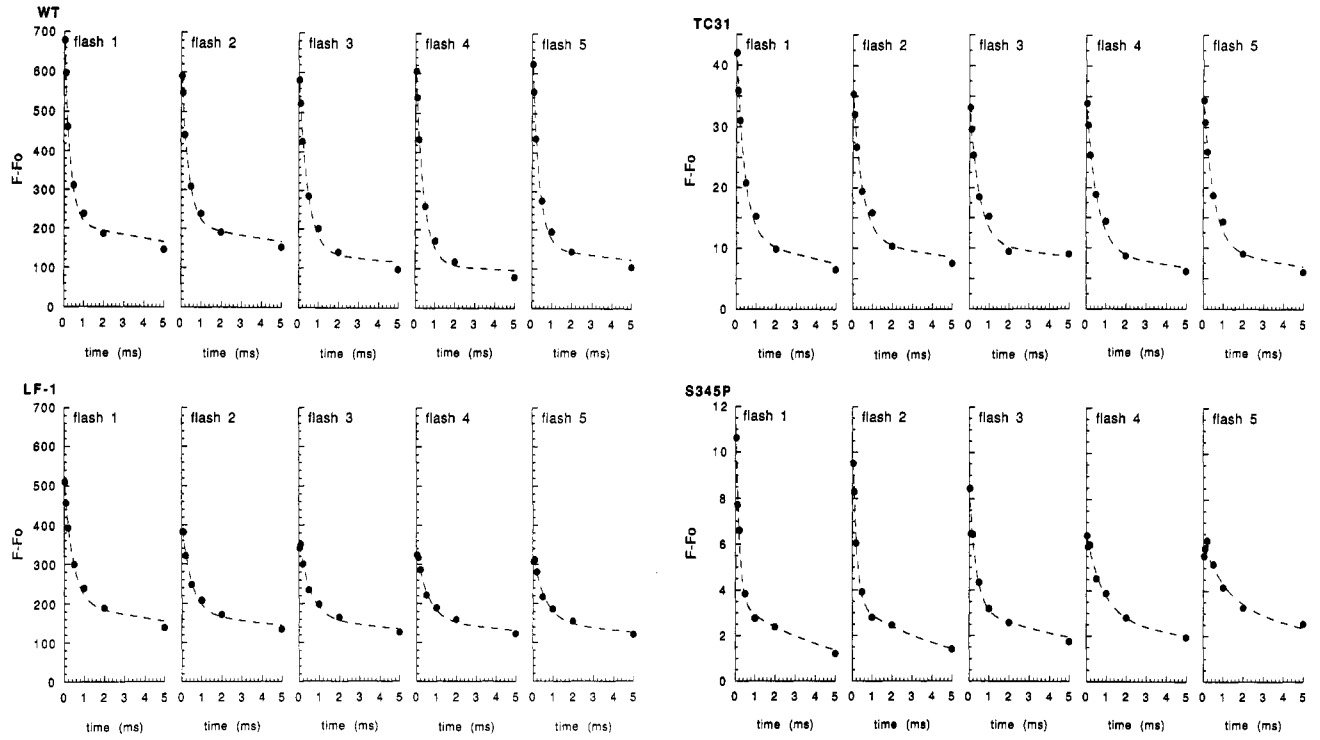

FIGURE 5: (a, left set of graphs) Relaxation of the variable fluorescence yield ($F - F_0$) after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole cells of *Scenedesmus* WT (upper) and LF-1 mutant (lower). Measurements are shown over a 5-ms range starting at 50 μs. The cells, suspended in NGY growth medium at 0.9 OD at 730 nm, were incubated for 10 min in the dark prior to the start of measurement. The 50-μs amplitudes of the variable fluorescence when normalized to F_0 as $(F - F_0)/F_0$ gave values following the first flash of 2.55 and 0.87 for *Scenedesmus* WT and LF-1, respectively. (b, right set of graphs) Relaxation of the variable fluorescence yield ($F - F_0$) after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole cells of *Synechocystis* 6803 TC31 (upper) and mutant D1 Ser345Pro (S345P, lower). The conditions are identical to those of part a except that the *Synechocystis* cells were suspended in BG-11 growth medium containing 5 mM glucose. The 50-μs values of the variable fluorescence normalized to F_0, $(F - F_0)/F_0$, following the first flash are 0.44 and 0.10 for *Synechocystis* TC31 and Ser345Pro, respectively.

state (F_0) despite reduction of Q_A. This is because P_{680}^+ quenches the fluorescence (Butler et al., 1973). The fluorescence yield then increases to the maximum level (F_{max}, $t_{1/2} \sim 50$ and 260 ns; Brettel et al., 1984; Mauzerall, 1972) with the reduction of P_{680}^+ by donor Z to form $Z^+P_{680}PheoQ_A^-Q_B$ (Gerken et al., 1988; van Best & Mathis, 1978). Reduction of Z^+ by the Mn cluster has little effect on the fluorescence yield; however, oxidation of Q_A^- by electron transfer to Q_B ($t_{1/2}$ = 100–200 μs; Robinson & Crofts, 1983) lowers the fluorescence yield again (approaching F_0) with the formation of the state $ZP_{680}PheoQ_AQ_B^-$.

To determine if a lack of processing of D1 were solely responsible for the lack of assembly of the Mn cluster, we compared, using this assay, the Ser345Pro mutant to the known nonprocessing mutant of *Scenedesmus*, LF-1 (Diner et al., 1988; Taylor et al., 1988b). It has been previously shown that this *Scendesmus* mutant lacks the protease responsible for C-terminal processing of precursor D1 (Taylor et al., 1988a). LF-1 is also completely inactive for oxygen evolution, making it non-photoautotrophic, and contains only 1–2 Mn/PSII reaction center (Metz et al., 1980), indicative of an inability to fully assemble the Mn cluster.

A comparison of the fluorescence yield at 50 μs after each of the actinic flashes is particularly revealing with regard to donor side function. Fifty microseconds is long compared to the rate of reduction of P_{680}^+ (see above) in the presence of an intact oxygen-evolving complex but short compared to the oxidation of the reduced primary plastoquinone electron acceptor, Q_A^-, by the oxidized secondary plastoquinone acceptor, Q_B (see above), or by Q_3^- ($t_{1/2}$ = 300-500 μs; Robinson & Crofts, 1983).

When the OEC is functional, as in the case of the *Scenedesmus* WT control shown in Figure 5a, the high-fluorescence state $P_{680}Q_A^-$ is apparent 50 μs after each of the flashes, giving rise to a small variation in fluorescence intensity with flash number consistent with the S-state dependence measured during the actinic flash lifetime by Delosme (1972). In contrast, the LF-1 mutant shows a marked and progressively increasing quenching at 50 μs on the second and subsequent flashes (Figure 5a). This result indicates that after the second and subsequent flashes, many of the reaction centers in this mutant show incomplete reduction after 50 μs of P_{680}^+, a quencher of chlorophyll fluorescence. This observation is indicative of a blockage in the reduction of Z^+.

A disruption on the donor side of the PSII complex is also indicated from an analysis, again using fluorescence, of the rate of charge recombination between the oxidized donor side and Q_A^-. This is done by giving a single actinic flash to intact cells in the presence of the herbicide DCMU, which blocks electron transfer from Q_A^- to Q_B, and following the relaxation of the fluorescence yield using weak probe flashes. Recombination is thought to occur between Q_A^- and P_{680}^+. The higher the equilibrated concentration of P_{680}^+ (the less stabilized the oxidizing equivalent) the faster the charge recombination. In *Scenedesmus* wild-type cells, after correction of the fluorescence relaxation for energy transfer, charge recombination is seen to occur between the S_2 state of the OEC and Q_A^- with a $t_{1/2} \sim 0.3$ s (Figure 6a). The correction for energy transfer is based on an assumed nonlinear dependence of fluorescence yield on $[Q_A^-]$ similar to that of *Chlorella* (Joliot et al., 1973). However, in the LF-1 mutant, charge recombination is clearly biphasic (Figure 6a), sug-

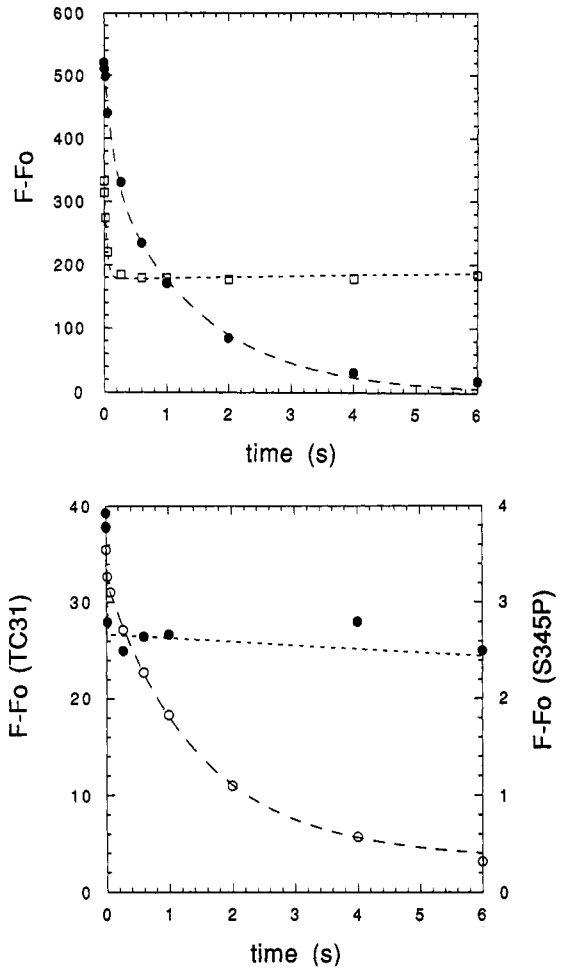

FIGURE 6: (a, top) Relaxation of the variable fluorescence yield ($F - F_0$) resulting from charge recombination between Q_A^- and the PSII donor side following a single saturating 2-μs flash excitation of whole cells of *Scenedesmus* WT (●) and LF-1 (□) in the presence of 30 μM DCMU. The cells were treated as in Figure 5a except that DCMU was added at least 1 min before the start of the measurement and the cells were suspended at an OD of 0.45 at 730 nm. The variable fluorescence normalized to F_0, $(F - F_0)/F_0$ at 500 μs, the earliest time point, is 1.52 and 0.53, respectively, for *Scenedesmus* WT and LF-1. (b, bottom) Relaxation of the variable fluorescence yield ($F - F_0$) resulting from charge recombination between Q_A^- and the PSII donor side following single saturating 2-μs flash excitation of whole cells of *Synechocystis* 6803 TC31 (○) and Ser345Pro (●, S345P) at an OD of 0.9 at 730 nm in the presence of 40 μM DCMU. The experimental conditions are otherwise the same as in Figure 5b. With the variable fluorescence normalized to F_0, $(F - F_0)/F_0$ at 500 μs, the earliest time point, is 0.36 and 0.04, respectively, for *Synechocystis* TC31 and Ser345Pro.

gesting a heterogeneity in the PSII centers. The small amount of fast phase ($t_{1/2}$ = 10–20 ms) observed is consistent with charge recombination between Z^+ and Q_A^- whereas the remaining slow phase ($t_{1/2} > 10$ s) is indicative of centers that have reduced Z^+ but by a donor other than the normal manganese cluster. A candidate for this alternative electron donor is free Mn^{2+}.

Figures 5b and 6b show the results obtained with the *Synechocystis* 6803 TC31 and Ser345Pro strains examined under the same conditions. Both the quenching of fluorescence in the five flash series and the kinetics of charge recombination following a single flash are reminiscent of the *Scenedesmus* WT and LF-1 mutant, respectively. The phenotypes of the LF-1 mutant, which lacks the processing protease activity, and the Ser345Pro mutant, which has an altered cleavage site, are therefore similar. Consequently, we conclude that a failure to process the D1 polypeptide does indeed block

Table I: Truncating the D1 Polypeptide Blocks Water Oxidation

mutant strain[a]	length of D1[b]	photosynthesis[c]	% PSII reaction center[d]	% oxygen evolution[e]
TC31	360/344	+	100	100
SΔ	344	+	~100	~100
Ala344stop	343	–	~40	0
Leu343stop	342	–	~25	0
Asp342stop	341	–	~25	0
Asn335stop	334	–	≤10	0

[a] See Materials and Methods for the construction of mutant strains. [b] Expressed in number of amino acids, assuming no N-terminal processing. [c] Measured in BG-11 medium according to Williams (1988). TC31 cells have a doubling time of 20 h when they are grown under photoautotrophic conditions. [d] Expressed as percent of TC31 on a per cell basis (OD 730). See Materials and Methods and Nixon and Diner (1992) for measurement conditions and an explanation of the experimental procedure. [e] Light-saturated rate expressed as a percent of TC31 on a per chlorophyll basis. See Materials and Methods and Nixon and Diner (1992) for the experimental procedure.

assembly of the manganese cluster and that the lack of processing is the sole reason for the lack of assembly of the photosynthetic manganese cluster in the LF-1 mutant.

Spontaneous Photoautotrophic Revertants of Mutant Ser345Pro. Eight spontaneous revertants of the Ser345Pro mutant were isolated that had regained the ability to grow photoautotrophically. In all cases, the *psbA3* gene amplified from the revertants through PCR could transform the original Ser345Pro mutant to photoautotrophy, indicating that the site of the reversion lay within the *psbA3* gene. The sequence of the C-terminal region of the D1 polypeptide was determined in each case by sequencing single-stranded DNA generated by asymmetric amplification of the *psbA3* gene. For one of the revertants, there had been a single base change so that a Ser codon was regenerated at position 345 (CCC → TCC). This revertant therefore restores the original wild-type residue at this position using a synonymous codon. In the other seven cases, however, there was an insertion of six bases (CTA GCG) between the codons specifying Ala344 and Pro345 so that the residues Leu and Ala were inserted into the the D1 polypeptide at this position. Consequently, Leu replaces Pro at the +1 position relative to the cleavage site (assuming faithful processing) and the C-terminal extension consists of 18 residues rather than the 16 residues found in WT. We therefore conclude that the length of the C-terminal extension of D1 is not crucial for assembly of a functional PSII complex. This reversion also eliminates Glu from the +3 position relative to the cleavage site. A carboxylate-containing amino acid is highly conserved at this position in the carboxy-terminal extension of D1 from many sources (Svensson et al., 1991). The successful processing in the insertional revertants implies the nonessential nature of a carboxylate-containing residue at this position.

The C-Terminal Residue of the Mature D1 Polypeptide Is Required for Assembly of a Functional Manganese Cluster. To investigate further the role of the C-terminus of the D1 polypeptide, we constructed a series of mutants in which the mature form of the D1 polypeptide was truncated at the C-terminus. Mutants Ala344stop, Leu343stop, Asp342stop, and Asn335stop synthesize D1 polypeptides that are shorter than the mature polypeptide by 1, 2, 3, and 10 residues, respectively. All of these mutants are incapable of evolving oxygen despite the presence of significant amounts of PSII (Table I). In addition, the shorter the length of the D1 polypeptide, the lower the concentration of PSII reaction centers that accumulate per cell (Table I). These results indicate a probable stabilization of the PSII reaction center

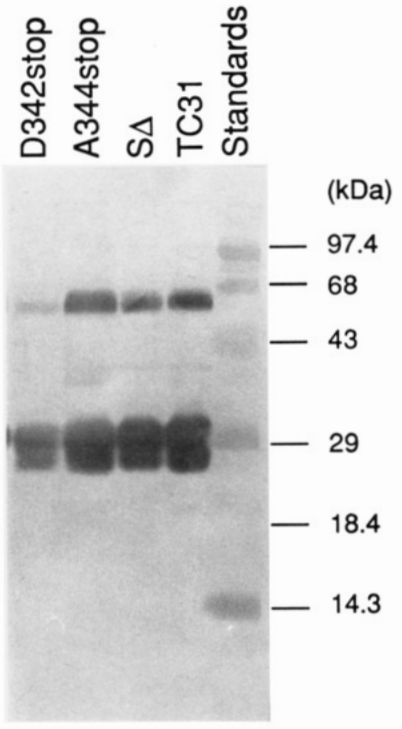

FIGURE 7: Immunoblot of PSII core complexes isolated from strains TC31, Asp342stop (D342stop), Ala344stop (A344stop), and SΔ using antiserum raised against the D1 polypeptide of spinach. The sizes of the prestained markers purchased from Baltimore Research Laboratories are shown on the right.

complex by the carboxy terminus of D1. The immunoblot shown in Figure 7 of PSII core complexes isolated from mutants Ala344stop and Asp342stop confirms, within the accuracy of this technique, that the D1 polypeptide has not been degraded to a more truncated form in these mutants. The site of disruption of electron transport within Ala344stop was determined by analyzing the decay in fluorescence intensity following each flash in a five-flash series as described above (Figure 8a) and the rate of charge recombination following a single saturating flash (Figure 8b). Using these criteria, the phenotype of mutant Ala344stop is similar to that of the *Scenedesmus* LF-1 and *Synechocystis* 6803 Ser345Pro mutants (Figures 5 and 6), indicating that without the C-terminal residue at position 344 the D1 polypeptide cannot assemble a functional manganese cluster.

Mutants S345P and A344stop Retain a High Affinity for Mn^{2+} in PSII Non-Oxygen-Evolving Core Complexes. We have shown in previous work that *Synechocystis* TC31 PSII core complexes lacking both Q_B and the OEC show reduction of Z^+ by exogenous Mn^{2+} with mixed first- ($K_d \leq 1\ \mu M$) and second-order components (second-order rate constant of (4–4.8) $\times 10^7\ M^{-1}\ s^{-1}$ (at pH 5.7; Diner & Nixon, 1992). In such complexes, there is competition between the charge recombination of Z^+ and Q_A^- and reduction of Z^+ by exogenous Mn^{2+}. A titration of the ability of Mn^{2+} to block charge recombination showed that at 1 μM Mn^{2+} (K_m) half of the centers are prevented from back-reacting (Nixon & Diner, 1992). The site at which Mn^{2+} is bound and oxidized is probably the same site at which the first Mn^{2+} is bound in the light-driven assembly of the cluster (Blubaugh & Cheniae, 1990; Nixon & Diner, 1992). We have shown that an important component of this site is the Asp170 residue of the D1 polypeptide (Nixon & Diner, 1992).

Figure 9a shows such an assay applied to core complexes isolated from *Scenedesmus* WT and LF-1 according to Diner et al. (1988). In wild type, charge recombination between Z^+ and Q_A^- was blocked in half of the centers at a concentration of 1 μM Mn^{2+}. In the case of the LF-1 mutant, the overall K_m is also 1 μM, but the shape of the titration curve differs slightly from that of WT, showing less of a higher affinity binding component.

Figure 9b shows a similar assay for *Synechocystis* TC31 and mutants Ser345Pro and Ala344Stop. As reported earlier (Nixon & Diner, 1992), TC31 shows a K_m for the reduction of Z^+ by Mn^{2+} of 1 μM. Mutants Ser345Pro and Ala344stop show similar values for this K_m, 1–2 μM and 2 μM, respectively, much lower than the K_m of ~50–60 μM observed for those mutants at the Asp170 position that are unable to evolve oxygen (i.e., Asp170Ser and Asp170Ala; Nixon and Diner, 1992). We therefore conclude that the high-affinity Mn^{2+} binding and oxidation site is still largely intact in mutants Ser345Pro and Ala344stop. However, as in the case of the WT *Scenedesmus* core complexes, there appears to be a small higher affinity component in *Synechocystis* TC31 that is diminished in the mutants.

The Nature of the C-Terminal Residue of Mature D1 Is Important for PSII Function. If the carboxy terminus is itself a ligand to the manganese cluster, then it is possible that there might be structural and/or charge constraints placed on the side chain of the C-terminal residue. This is supported by the fact that Ala344 is totally conserved in all the D1 sequences so far determined (Svensson et al., 1991). We have examined the effect on PSII function of substituting Ala344 by the residues Gly, Met, Ser, Val, Tyr, and Lys. These mutants were all constructed in the SΔ background (i.e., lacking the C-terminal extension) so that their phenotypes were not complicated by potential problems in the processing of the D1 precursor polypeptide. All the mutants apart from the Tyr and Lys changes are able to grow photoautotrophically. Mutants Ala344Lys and Ala344Tyr evolve oxygen, at light-saturated rates that are ~10% and ~20% of WT levels, respectively. These rates are to be compared to levels of PSII reaction centers that are 15–20% and 70–80% of TC31 for the Ala344Lys and Ala344Tyr mutants, respectively, indicating that of the two the presence of the bulky tyrosine appears to be more of an impediment either to successful assembly of the Mn cluster or to water oxidation.

DISCUSSION

In the first part of this paper, we describe how we construct mutants in the *psbA3* gene of *Synechocystis* 6803. The use of this copy of the *psbA* gene family for constructing mutants has been complicated in previous work (Metz et al., 1989) by the presence of an open reading frame starting 150 bp downstream of the stop codon for *psbA3* which shows homology to the *dnaE* gene of *Bacillus subtilis*. Interruption of this ORF (designated ORF X, Figure 1) by the spectinomycin-resistance cassette from pHP45Ω leads to strains that exhibit incomplete segregation of the $spec^R$ gene among the approximate 10 copies of the chromosome (Metz et al., 1989). This incomplete segregation has been interpreted as evidence in favor of a crucial role for ORF X in cell growth (Metz et al., 1989). An examination of the nucleotide sequence of the spacer region between ORF X and *psbA3* could not identify any recognizable prokaryotic-like promoter sequences—raising the possibility that *psbA3* and ORF X were cotranscribed in vivo. However, replacement of the *psbA3* gene in deletion strains TD34 and TD41 with the spectinomycin-resistance cassette from pHP45Ω, which acts as a terminator of transcription (Prentki & Krisch, 1984), argues against this

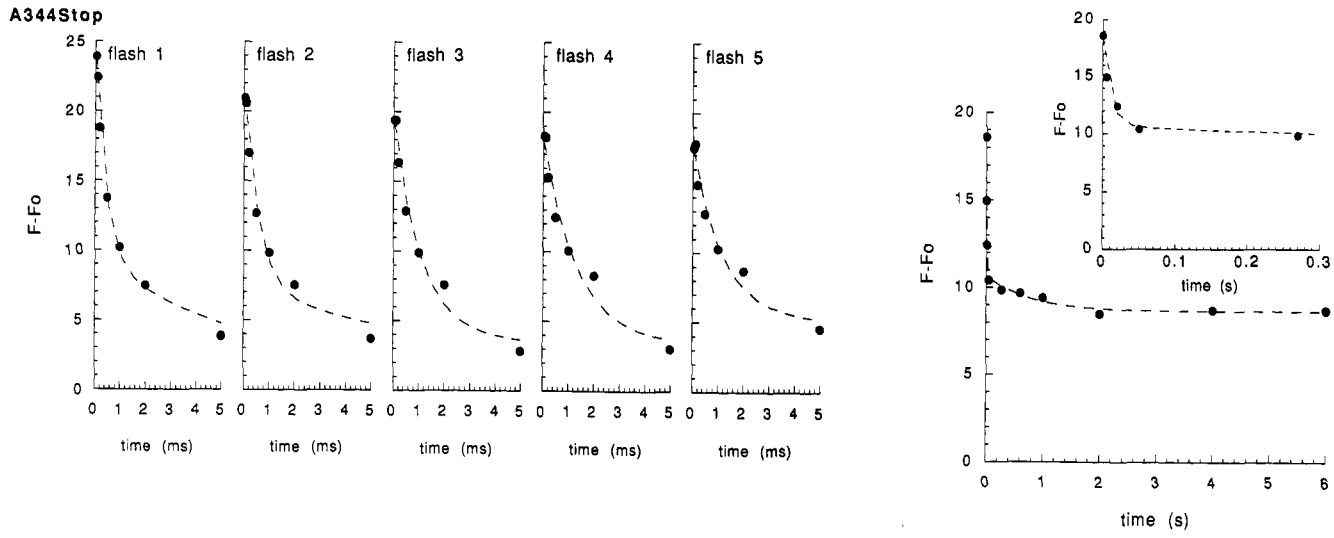

FIGURE 8: (a, left set of graphs) Relaxation of the variable fluorescence yield ($F - F_0$) after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole cells of *Synechocystis* 6803 mutant Ala344stop. The conditions of the measurement are the same as in Figure 5b. The 50-μs value of the variable fluorescence normalized to F_0, $(F - F_0)/F_0$, following the first flash is 0.24. (b, far right graph) Relaxation of the variable fluorescence yield ($F - F_0$) resulting from charge recombination between Q_A^- and the PSII donor side following a single saturating 2-μs flash excitation of whole cells of *Synechocystis* 6803 mutant Ala344stop. The conditions of the experiment are the same as those of Figure 6b. With the variable fluorescence normalized to F_0, $(F - F_0)/F_0$ at 500 μs, the earliest time point, is 0.175. The insert shows the earlier time points on an expanded scale.

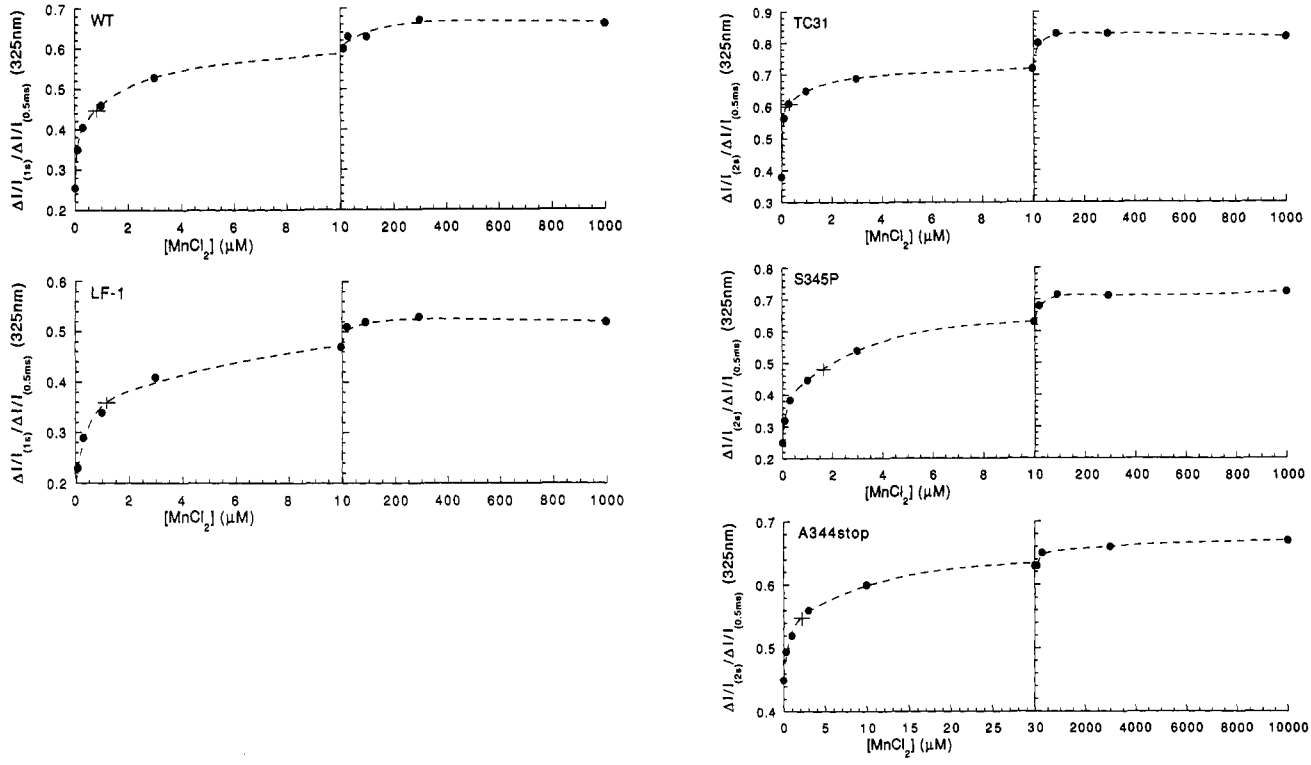

FIGURE 9: (a, left set of graphs) The concentration dependence of the ability of exogenous Mn^{2+} to block the reoxidation of Q_A^- by charge recombination in non-oxygen-evolving PSII core complexes of *Scenedesmus* WT (upper) and mutant LF-1 (lower) following a 2-μs actinic flash. The ordinate scale is the ratio of the concentration of Q_A^- (measured at 325 nm) at 1 s after the actinic flash to that at 0.5 ms. PSII core complexes (10 μM chlorophyll) were suspended in 20 mM MES–NaOH (pH 5.7), 1 mM $CaCl_2$, and 1 μM $K_3Fe(CN)_6$. The plus sign indicates the concentration of Mn^{2+} that blocks half of the centers from recombining (overall K_m). (b, right set of graphs) The concentration dependence of the ability of exogenous Mn^{2+} to block the reoxidation of Q_A^- by charge recombination in non-oxygen-evolving PSII core complexes of *Synechocystis* TC31 (upper) and mutants D1 Ser345Pro (middle) and Ala344stop (lower) following a 2-μs actinic flash. The ordinate scale is the ratio of the concentration of Q_A^- (measured at 325 nm) at 2 s after the actinic flash to that at 0.5 ms. The conditions are otherwise the same as in part a.

possibility. Our results therefore indicate the presence of a promoter downstream of the *Hin*cII site into which the spectinomycin cassette was inserted in TD34, some 54 bp upstream of the putative initiation codon of ORF X. Interestingly, 40 bp upstream from the initiation codon of ORF X is the nucleotide sequence CCCCAT, identified recently as a possible consensus sequence for a heat-shock promoter in *Synechocystis* (Chitnis & Nelson, 1991).

In the second part of this paper, we employ this mutagenesis system to probe the mechanism of C-terminal processing of D1 and its role in the assembly of the photosynthetic manganese cluster. We have confirmed by sequencing the C-terminal

tryptic peptide of D1 that C-terminal processing of the D1 polypeptide does indeed occur within the cyanobacterium *Synechocystis* 6803 and that the site of cleavage, on the carboxy side of Ala344, is identical to that found in higher plants (Takahashi et al., 1988, 1990) and *Chlamydomonas* (B. A. Diner, unpublished data). This conclusion is also supported by the ability of an antibody raised against the C-terminal extension (residues Ser345–Gly360) of D1 to cross-react with mutant Ser345Pro (whose D1 is unprocessed) and a lack of cross-reaction with TC31 (Figure 4). Thus, with the exception of the green alga *Euglena gracilis*, which apparently lacks a C-terminal extension (Karabin et al., 1984; Keller & Stutz, 1984), C-terminal processing of D1 appears to be ubiquitous in nature.

The function of the extension is unknown although it has been speculated that it is required for insertion of D1 into the thylakoid membrane (Marder et al., 1984) or for incorporation of D1 into the PSII complex (Taylor et al., 1988a). To address these possibilities, we constructed a mutant, designated SΔ, which lacks the 16 amino acid extension at the C-terminus. Despite the absence of the extension, this mutant could still evolve oxygen and grow photoautotrophically at wild-type rates, indicating little perturbation to the assembly of either the PSII reaction center or the oxygen-evolving manganese cluster. We have so far been unable to identify conditions that allow the WT to grow significantly faster than SΔ. If the C-terminal extension is of functional significance, then it would appear to lie outside the assembly of D1 into a functional PSII complex. While the SΔ and Ser345stop mutations were first introduced into *Synechocystis* 6803 [see Diner et al. (1991b) for a preliminary report], mutation D1 Ser345stop has now been introduced into *Chlamydomonas* with similar results (Schrader & Johanningmeier, 1992; Lers et al., 1992). Possible roles for the extension and its processing may include a regulation of photosynthetic activity, a protection against proteolysis of centers that have not yet assembled the manganese cluster, or perhaps a targeting function to direct the D1 polypeptide exclusively to the thylakoid membrane. These possibilities are being investigated.

The specificity behind the processing reaction is as yet unknown. Recent work has demonstrated that the processing protease found in *Synechocystis* 6803 can recognize and process a D1 precursor polypeptide from a higher plant source despite the considerable differences in size and sequence between the two C-terminal extensions (Nixon et al., 1991). This would suggest that the primary structure of the C-terminal extension is not the major determinant for the recognition of D1 by the protease. This conclusion is also supported by the isolation of photosynthetic revertants of mutant Ser345Pro which contain an insertion of two amino acid residues within the extension. However, we have shown in this paper that the nature of the residue in the +1 position relative to the cleavage site is important for cleavage. When it is changed to Pro, cleavage does not occur. We have not yet determined whether this is as a result of a failure of the protease to bind the substrate or whether the resulting Ala–Pro bond is resistant to proteolysis. Processing still occurs, however, when Ser345 is replaced by either the similarly-sized alanine residue (which is the residue found in higher plants at this position) or the charged and relatively bulky residue Arg. Leu345 also apparently works, judging from the photoautotrophy of the Ser345Pro insertional revertants. These observations are reminiscent of the cleavage of the signal sequence of secreted proteins where it has been found that a wide spectrum of residues can be tolerated at the +1 position [e.g., Borchert and Nagarajan (1991)] with the sole exception of proline (Nothwehr et al., 1990; von Heijne, 1986).

From our mutagenesis studies, we have identified two classes of D1 mutant that are unable to evolve oxygen. The first, exemplified by mutants at Asp170, has a manganese-binding site, associated with the PSII core complex, with a lowered affinity for Mn^{2+} (Nixon & Diner, 1992; Diner & Nixon, 1992). The Mn cluster is assembled in a fraction of PSII centers in the less perturbed of this class of mutants and not at all in those that are completely inactive for O_2 evolution. In the latter mutants D1 Tyr161, Z is the terminal electron donor of PSII (Nixon & Diner, 1992).

The second class of mutant encompasses residues within the carboxy-terminal region of the mature D1 polypeptide, at His332, Asp342 (P. J. Nixon and B. A. Diner, manuscript in preparation), and Ala344, as well as mutants that block processing of D1 (Ser345Pro and *Scenedesmus* LF-1). The manganese cluster is incompletely assembled, but in contrast to the first class of mutant the high-affinity manganese binding site remains largely intact. Thus, Mn^{2+} is able to bind to the PSII complex in these mutants and reduce Z^+ in single-turnover experiments. This probably explains the extremely slow phase in the rate of charge recombination between Q_A^- and the oxidized donor side (Figures 6 and 8b).

At present, we are unable to accurately quantitate the amount of manganese associated with PSII complexes from mutants of *Synechocystis* 6803, a problem encountered by other investigators [e.g., Vermaas et al. (1990)], and so we have been unable to determine the number of Mn assembled into the OEC of these mutants. However, a technique of some promise for *Synechocystis* 6803 membranes and core complexes has recently been reported by Boerner et al. (1992). For the LF-1 mutant of *S. obliquus*, Metz and co-workers have determined a stoichiometry of 1–2 Mn ions/PSII reaction center (Metz et al., 1980). Thus, the phenotype of the second class of mutants described above is consistent with binding of a single Mn ion at the high-affinity site and an inability to assemble the remainder of the cluster. However, we cannot rule out the possibility that a fraction of the centers, in some of the class II mutants, have assembled an intact cluster but that the redox properties are modified such that the S-states are unable to advance.

Recently, Seibert and co-workers have studied a high-affinity manganese-binding site of PSII that is responsible for inhibiting 1,5-diphenylcarbazide- (DPC-) supported photoreduction of 2,6-dichlorophenolindophenol (DCIP) (Seibert et al., 1989; Preston & Seibert, 1989, 1991a,b). These authors suggest that the original high-affinity site identified by Hsu et al. (1987) is in fact composed of four equivalent sites (Preston & Seibert, 1991b). It is proposed that LF-1 lacks half of the original high-affinity manganese-binding sites (Seibert et al., 1989) so that only two of the four sites identified with this assay (Preston & Seibert, 1991b) are present. Possibly one of these remaining sites is identical to the high-affinity site described in this paper and elsewhere (Nixon & Diner, 1992). However, an underlying problem with the assay employed by Hsu et al. and by Seibert and co-workers is that there is no evidence to link their particular high-affinity site with sites involved in the photoactivation process. Furthermore, the assay, performed under light-saturated conditions, is probably limited by the rate of dissociation or reduction of Mn^{3+} and by the rate of turnover of the reaction center, most likely determined by the rate of reoxidation of Q_A^-. The single-flash assay system that we use here (Figure 9; Nixon & Diner, 1992) has none of these drawbacks. The Mn^{2+} binding and

oxidation site identified using our assay system appears to be functionally significant because (i) Mn^{2+} bound at this site has been shown to reduce Z^+ (Nixon & Diner, 1992; Diner & Nixon, 1992), (ii) the K_m of ~ 1 μM for Mn^{2+} photooxidation is close to the value proposed for the oxidation of Mn^{2+} in the first step of photoactivation (Blubaugh & Cheniae, 1990), and (iii) mutants at Asp170 of the D1 polypeptide that are unable to evolve oxygen (Nixon & Diner, 1992) show an increase in the K_m for Mn^{2+} oxidation at this site. However, we find that the class II mutants, including *Scenedesmus* LF-1, assayed using our technique show some small loss of a high-affinity component despite the similarity to WT in the overall K_m (1–2 μM). There is, therefore, some agreement between our results and those of Preston and Seibert (1991b) concerning the role for the carboxy terminus in high-affinity binding of Mn^{2+}. Furthermore, as we have already shown that D1 Asp170 is implicated in a high-affinity Mn^{2+}-binding site, a perturbation of such binding by mutation at the C-terminus would imply an interaction between the two—i.e., a folding back of the C-terminus toward Asp170 in the loop connecting transmembrane helices III to IV [see also Diner et al. (1991b)].

We report here that oxygen evolution is blocked in a mutant (Ala344stop) that synthesizes a D1 polypeptide shortened by one residue when compared to the mature form of wild-type D1. This result again emphasizes the importance of the C-terminus of D1 in assembling a functional Mn cluster. A simple model to explain the behavior of this mutant is that the free carboxyl group at the C-terminus of the mature D1 polypeptide is itself a ligand to the Mn cluster. Such a scenario also explains why *Scenedesmus* LF-1 and *Synechocystis* D1 Ser345Pro cannot assemble a functional cluster, as in these mutants the C-terminal carboxyl group at position 344 is implicated in a peptide bond and is consequently unavailable for ligation of Mn.

Similar C-terminal truncation mutants have been constructed in the D2 polypeptide (D. Chisholm, personal communication) and the α subunit of the cytochrome *b*559 apoprotein (Tae & Cramer, 1992). In contrast to the mutants reported here, an intact manganese cluster could still be assembled in both cases.

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