Evidence from Directed Mutagenesis That Aspartate 170 of the D1 Polypeptide Influences the Assembly and/or Stability of the Manganese Cluster in the Photosynthetic Water-Splitting Complex†

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ABSTRACT: To identify amino acid residues that influence the assembly or stability of the manganese cluster in photosystem II, we have generated site-directed mutations in the D1 polypeptide of the cyanobacterium, *Synechocystis* sp. PCC 6803. Indirect evidence has suggested that the D1 polypeptide provides some of the ligands that are required for metal binding. Mutations at position 170 of D1 were selected for characterization, since an aspartate to asparagine mutation (DN170D1) at this position completely abolishes photoautotrophic growth, while retention of a carboxylic acid at this position (aspartate to glutamate, DE170D1) supports photoautotrophic growth. Photosystem II particles were purified from control, DE170D1, and DN170D1 cells by a procedure that retains high rates of oxygen evolution activity in control particles [Noren, G. H., Boerner, R. J., & Barry, B. A. (1991) *Biochemistry 30*, 3943–3950]. Spectroscopic analysis shows that the tyrosine radical, Z^+, which normally oxidizes the manganese cluster, is rapidly reduced in the DE170D1 mutant, but not in the DN170D1 mutant. A possible explanation of this block or dramatic decrease in the rate of electron transfer between the manganese cluster and tyrosine Z is an alteration in the properties of the metal center. Quantitation of manganese in these particles is consistent with aspartate 170 influencing the stability or assembly of the manganese cluster, since the aspartate to asparagine mutation results in a decrease in the manganese content per reaction center. Photosystem II particles from DN170D1 show a 60% decrease in the amount of specifically bound manganese per reaction center, when compared to control particles. Also, we observe a 70% decrease in the amount of specifically bound manganese per reaction center in partially purified DN170D1 particles and at least an 80% decrease in the amount of hydroxylamine-reducible manganese in DN170D1 thylakoid membranes. Single-turnover fluorescence assays and steady-state EPR measurements demonstrate that the remaining, endogenous manganese does not rapidly reduce tyrosine Z^+ in the DN170D1 mutant. Additional evidence that aspartate 170 influences the assembly or stability of the metal site comes from analysis of the DE170D1 mutant. Although this mutant assembles a functional manganese cluster, as assessed by oxygen evolution and spectroscopic assays, the properties of the manganese site are perturbed.

In oxygenic photosynthesis, which occurs in plants, green algae, and cyanobacteria, two chlorophyll-containing photosystems cooperate to transfer electrons from water to $NADP^+$. Photosystems I and II are located in the thylakoid membrane of chloroplasts and cyanobacteria. Energy derived from light absorption is used to drive the electron-transfer reactions and to generate a proton gradient across the thylakoid membrane. As a byproduct of water oxidation, molecular oxygen is produced. Photosystem II is the reaction center that catalyzes the light-driven oxidation of water and the reduction of bound quinone.

Photosystem II is composed of both intrinsic, hydrophobic and extrinsic proteins, as well as the cofactors that are necessary for light-driven charge separation. Some of the components necessary for maximal oxygen evolution are the D1 and D2 integral membrane polypeptides; the CP47 and CP43 integral membrane polypeptides, which are believed to serve as a tightly bound antenna; cytochrome b_{559}; and the 33-kDa extrinsic protein, which stabilizes the environment of the manganese cluster [for reviews, see Babcock (1987) and Debus (1992)]. D1 and D2 are believed to form the heterodimer core of the reaction center and to bind the cofactors that are involved in the light-driven charge separation.

Upon light absorption, the primary chlorophyll donor of photosystem II, P_{680},[1] reduces a bound pheophytin, which in turn reduces a bound plastoquinone molecule, Q_A. Q_A^- reduces a second plastoquinone, Q_B; this quinone, unlike Q_A, functions as a two-electron gate. A redox active tyrosine residue, Z, is oxidized by the cation radical of the primary donor, P_{680}^+. The EPR-detectable tyrosine radical, Z^+, is then rapidly reduced by a metal cluster that is composed of four manganese atoms. This manganese cluster is the catalytic site of water oxidation and accumulates four oxidizing equivalents as

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[1] Abbreviations: chl, chlorophyll; DCBQ, 2,6-dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; P_{680}^+, primary chlorophyll donor of photosystem II; P_{700}^+, primary chlorophyll donor of photosystem I; PCR, polymerase chain reaction; PSI, photosystem I; PSII, photosystem II; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TM, thylakoid membranes; Tris, tris(hydroxymethyl)aminomethane.

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sequential metal- or ligand-centered oxidations before the release of molecular oxygen. The manganese cluster cycles through a series of five oxidation states that are referred to as S states, S_0–S_4. In the dark, the majority of the sample is present in the S_1 state (Babcock, 1987; Debus, 1992). Recent evidence suggests that the manganese cluster may also contain calcium [for a review, see Debus (1992)].

In addition to Z, there is another redox-active tyrosine residue in photosystem II that forms a stable free radical, D^+ (Commoner et al., 1956; Barry & Babcock, 1987). The EPR line shape of D^+ is identical to that of Z^+ (Hoganson & Babcock, 1988). The physiological importance of D^+ is unclear. Analysis of site-directed mutants suggests that D is tyrosine 160 of the D2 polypeptide and that Z is tyrosine 161 of D1 (Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989; Noren & Barry, 1992).

While the manganese cluster is absolutely necessary for water oxidation, little is known regarding its location. Several models for its location have been proposed based on sequence homology with reaction centers of purple non-sulfur bacteria (Coleman & Govindjee, 1987; Dismukes, 1988; Svensson et al., 1990, 1991). There are five lines of indirect experimental evidence that suggest that amino acid residues in the D1 polypeptide may influence the stability or assembly of the manganese cluster [for a review, see Debus (1992)]. First, microwave power saturation studies (Warden et al., 1976; Yocum & Babcock, 1981; Yocum et al., 1981; Rutherford et al., 1988; Styring & Rutherford, 1988; Isogai et al., 1988; Innes & Brudvig, 1989; Babcock et al., 1989; Beck et al., 1990) and spin lattice relaxation time measurements (de Groot et al., 1986; Britt et al., 1987; Evelo et al., 1989) imply close proximity between the tyrosine residue, Z, of the D1 polypeptide and manganese. Second, a mutant of *Scenedesmus obliquus* that has a defect in the processing of the carboxyl end of the D1 polypeptide is incapable of binding functional manganese (Metz et al., 1980; Metz & Bishop, 1980; Rutherford et al., 1988; Diner et al., 1988; Seibert et al., 1988; Taylor et al., 1988a,b). Third, a correlation between degradation of the D1 polypeptide and loss of bound manganese has been observed (Virgin et al., 1988). Fourth, specific, light-induced, iodination of Cl-depleted PSII membranes is specific to the D1 polypeptide (Ikeuchi et al., 1988; Ikeuchi & Inoue, 1988). Fifth, chemical modification and proteolytic digestion studies have implicated carboxylate and histidine residues from the D1 polypeptide as potential manganese ligands (Tamura et al., 1989; Seibert et al., 1989; Preston & Seibert, 1991a,b).

Several lines of evidence suggest that carboxylate residues, such as glutamate and aspartate, are good candidates to be manganese ligands. Spectroscopic studies have shown that the manganese cluster is ligated predominantly by oxygen atoms (Britt et al., 1987, 1989, 1990; DeRose et al., 1991). Model studies of manganese clusters in vitro suggest that some of these coordinating oxygens must be contributed by carboxylate residues in order to destabilize high oxidation states of the metal cluster and to facilitate catalysis (Pecoraro, 1988; Brudvig & Crabtree, 1989). Also, in a study utilizing site-directed mutagenesis of the D2 polypeptide, glutamate 69 of the D2 polypeptide was suggested to be one of several carboxylate groups that ligate manganese (Vermaas et al., 1990).

Taken together, the arguments presented above suggest that glutamate and aspartate residues in the D1 polypeptide may provide ligands to the manganese cluster. To identify these ligands or, more generally, to identify residues that influence the assembly or stability of the manganese cluster, we have generated site-directed mutations in the D1 polypeptide of the cyanobacterium *Synechocystis* sp. PCC 6803. This organism can grow photoheterotrophically in the presence of glucose, allowing for the growth and analysis of mutants that are not able to grow photoautotrophically (Williams, 1988). Mutations were constructed at carboxylic acid residues of D1 that are conserved in more than 31 organisms (Svensson et al., 1990, 1991) and that are located in the predicted lumenal hydrophilic loops (Trebst, 1986). Mutations at aspartate 170 were chosen for further study, since position 170 was one of two sites where elimination of a carboxylic acid abolished photoautotrophic growth (Debus et al., 1991). Aspartate 170 has also been identified as a potential ligand through analysis of an independently constructed set of D1 mutations in *Synechocystis* sp. PCC 6803 (Nixon & Diner, 1991). By analyzing a series of mutations constructed at position 170, these authors have concluded that aspartate 170 is critical for the function and assembly of the manganese cluster (Nixon & Diner, 1990, 1992). A non-oxygen-evolving photosystem II preparation, which presumably contains no functional manganese, was used to characterize these mutants (Diner et al., 1990; Nixon & Diner, 1990, 1992). Here, we use an oxygen-evolving photosystem II preparation (Noren et al., 1991) that retains functional manganese in order to characterize two mutations constructed at position 170 of the D1 polypeptide. On the basis of our biochemical and spectroscopic characterization of an aspartate to glutamate (DE170D1) and an aspartate to asparagine (DN170D1) mutation, we conclude that aspartate 170 plays an important role in stabilization or assembly of the manganese cluster.

MATERIALS AND METHODS

Construction and Confirmation of Mutants. Mutations were constructed as previously described by Debus et al. (1988b) and were introduced into a strain of the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803, that lacks all three of its *psb*A genes (Debus et al., 1990). The oligonucleotides 5′-GGTCAAGGaTCCTTCTCTGAGGGTATG-CCC-3′, 5′-CCTTCTCTAATGGcATGCCCTTGGG-3′, and 5′-GGCTCCTTCTCTGCAGGTATGCCCTTGGG-3′ were synthesized to create the aspartate to glutamate (DE170D1), aspartate to asparagine (DN170D1), and aspartate to alanine (DA170D1) mutations, respectively. The bases in lower case letters are those that introduced these mutations. The boldfaced bases are additional "silent" mutations that were introduced to create convenient sites for restriction endonucleases (*Bam*HI for DE170D1 and *Sph*I for DN170D1) without changing the amino acid sequence. The two-base mutation that changed aspartate to alanine introduced a site for *Pst*I. Following transformation into *Synechocystis* 6803, mutants were selected as described by Debus et al. (1990). The wild-type* strain used as a control in this study was obtained by identical procedures, except that the transforming plasmid carried no mutation (Debus et al., 1990).

To confirm that homologous recombination had taken place, genomic DNA from each mutant was isolated, digested with *Xba*I, and analyzed on Southern blots as described by Debus et al. (1990). To confirm the identity of each mutant and to verify the absence of unintentional mutations, a 1380-bp fragment of genomic DNA that includes the entire *psb*A-2 coding region was amplified by PCR, and the complete DNA sequence of *psb*A-2 was obtained by direct sequencing of the double-stranded PCR product. Further details of these procedures will be described in a forthcoming manuscript (Chu

et al., in preparation). To verify that no mutations outside of the *psb*A-2 coding region contributed to the loss of photoautotrophic growth in DN170D1 or DA170D1, cells of these mutants were transformed with a cloned fragment of the wild-type *psb*A-2 gene (the 594-bp *Bst*EII/*Bst*EII fragment) that includes the Asp-170 codon. Transformants were selected for photoautotrophic growth. For both DN170D1 and DA170D1, photoautotrophic transformants were recovered at frequencies that were several hundredfold higher than the frequency at which spontaneous revertants appeared.

In order to show that the second, silent mutation introduced into each of the DN170D1 and DE170D1 strains had no effect on the phenotype, the *psb*A-2 coding regions from photoautotrophic (PS+) revertants of both DN170D1 and DA170D1 were amplified by PCR and sequenced. Photoautotrophic revertants of DN170D1 were found to have retained the mutation that introduced the site of *Sph*I, but had spontaneously changed the Asn-170 codon, AAT, to the Asp codon, GAT. This result demonstrates that the silent mutation does not give rise to the non-photoautotrophic (PS–) phenotype of the DN170D1 mutant. A photoautotrophic revertant of DA170D1 was found to have spontaneously changed the Ala-170 codon GCA to GAA and, hence, had become an alternate form of DE170D1; a form that does not contain a silent mutation site. The oxygen evolution and fluorescence characteristics of this spontaneous revertant of DA170D1 were indistinguishable from those of the DE170D1 strain that is described in this manuscript, demonstrating that the silent mutation that introduced the site for *Bam*HI in DE170D1 was not responsible for the phenotype of this mutant.

Growth of Cells. *Synechocystis* 6803 cells were grown as described by Noren et al. (1991) in BG-11 media that was supplemented with 5 mM glucose and 5 mM TES–NaOH, pH 8.0. Cells of DN170D1 were harvested after 7 days because the PSI to PSII ratio increased after this point. Cells of DA170D1 were harvested after 5–6 days because the apparent PSII content of these cells decreased dramatically after this time. Approximately 100 L of each of the wild-type*, DE170D1, and DN170D1 cells was used in these studies. Cells of DN170D1 were found to have a nonnegligible rate of spontaneous reversion. Therefore, as a precaution, an aliquot was reserved for genomic DNA isolation from all of the 15-L cultures that were used for membrane and photosystem II isolation. The complete *psb*A-2 sequence was obtained following PCR amplification of DNA that was isolated from the pooled DN170D1 aliquots. No traces of the wild-type* Asp-170 codon or of any other spontaneous mutation within *psb*A-2 (e.g., second site revertants) were detected.

[^{14}C]DCMU Binding Assays. Quantitation of PSII on a chlorophyll basis in whole cells was performed as described by Vermaas et al. (1990) with minor modifications. Cells (25 μg of chlorophyll in 1 mL of growth media) were incubated for 30–60 min in darkness in the presence of 5–100 nM [^{14}C]-DCMU (243 μCi/mg; Amersham, kindly supplied by W. F. J. Vermaas). To control for nonspecific or nonphotosystem II binding of DCMU, duplicate samples containing [^{14}C]-DCMU were incubated in the presence of 20 μM atrazine. All samples contained 0.6% methanol. After incubation, the samples were centrifuged at 14000*g* for 2 min, and 0.8 mL of each supernatant was mixed with 10 mL of Beckman Ready-Safe scintillation cocktail. The ^{14}C levels were determined with a Beckman LS-3801 liquid scintillation counter. The instrumental counting efficiency, determined with a Beckman unquenched standard, was 98%. The counting efficiency was lowered by an additional 1% when 0.8 mL of growth medium was added to solid [^{14}C]DCMU dissolved in 10 mL of scintillation cocktail. For each DCMU concentration, the amount of bound [^{14}C]DCMU was determined by subtracting the amount of free DCMU in samples without atrazine from the amount in samples with atrazine.

Purification of Photosystem II Particles. PSII particles were purified from whole cells as in Noren et al. (1991) with the following modifications. For the Fast-flow Q column, a 400-mL gradient was started immediately after loading the column. DN170D1 mutant fractions from the ion-exchange columns had more contaminating PSI than wild-type* or DE170D1 fractions, and as a result, fewer fractions were collected from the DN170D1 Mono Q column. PSII particles from each cell type were resuspended in a buffer containing 50 mM MES–NaOH, pH 6.5, 20 mM $CaCl_2$, 15 mM NaCl, 0.05% (w/v) lauryl maltoside, and 50% (v/v) glycerol, pooled, and frozen at –80 °C.

Oxygen Evolution Assays, SDS–PAGE, Western Analysis, and Optical Quantitation of P_{700}^+ and Cytochrome b_{559}. These procedures were performed as described previously (Noren et al., 1991). The oxidized minus reduced difference spectrum was obtained for cytochrome b_{559} quantitation. Ferricyanide (2 mM) was used as the oxidant; either 4 mM dithionite or 2 mM ascorbate was used as the reductant. An extinction coefficient of 21.5 mM^{-1} cm^{-1} (559 – 577 nm) (Cramer et al., 1986) was used. Absorption maxima in the mutant *and* wild-type* difference spectra were 559 – 560 nm, when either ascorbate or dithionite was used as the reductant. When ascorbate was used as a reductant, the bandwidth at half-maximal amplitude was 11, 12, and 11 nm for wild-type*, DE170D1, and DN170D1 preparations, respectively. When dithionite was used as the reductant, the bandwidth at half-maximal amplitude was 15, 12, and 11 nm for wild-type*, DE170D1, and DN170D1, respectively. The narrow, symmetric line shape, bandwidth, and absorption maxima of the mutant difference spectra imply that there is little cyt b_6/f contamination in the mutant preparations (Cramer & Whitmarsh, 1977). The wild-type* difference spectrum is slightly asymmetrical when dithionite is used as a reductant, which may be caused by a small amount of cytochrome *f* contamination (Ho & Krogmann, 1980).

Manganese Quantitation. Manganese quantitation in photosystem II particles, partially purified, Fast-flow Q material, and thylakoid membranes was performed using HCl, as described previously (Noren et al., 1991). After elution in high NaCl from the second anion-exchange column and subsequent PEG precipitation (Noren et al., 1991), PSII particles were resuspended in a buffer containing 50 mM Mes–NaOH, pH 6.5, 20 mM $CaCl_2$, 15 mM NaCl, 0.05% (w/v) lauryl maltoside, and 50% (v/v) glycerol and then assayed for manganese. The same buffer, but with only 25% glycerol, was used for manganese quantitation of partially purified samples, which were eluted in high $MgCl_2$ from the first anion-exchange (Fast-flow Q) column and then PEG precipitated. Thylakoid membranes were suspended in the buffer described below.

For manganese quantitation in thylakoid membranes, hydroxylamine was used as a reductant in an attempt to observe only the high-oxidation-state manganese ions that are associated with photosystem II (Mei & Yocum, 1991; Yocum et al., 1981). Thylakoid membranes, at a chlorophyll concentration of 0.3 mg/mL, were suspended in a buffer containing 50 mM Mes–NaOH, pH 6.0, 40 mM $CaCl_2$, 20 mM $MgCl_2$, 150 mM NaCl, and 25% glycerol. The membranes were

Table I: Comparison of Rate of Oxygen Evolution [μmol of O_2 (mg of chl)$^{-1}$ h^{-1}] in Wild Type* and Mutants[a]

	cells[b]		TM[c]		PSII[c]	
	-DCMU	+DCMU (% inhib)	-DCMU	+DCMU (% inhib)	-DCMU	+DCMU (% inhib)
wild type*[g]	620[e]	80%[d]	520	70%[d]	2200[f]	70%[d]
DE170D1[g]	340[e]	70%[d]	330	60%[d]	640[f]	70%[d]
DN170D1	<50					

[a] Cells and thylakoid membranes were assayed with 1 mM potassium ferricyanide and 1 mM DCBQ, while PSII particles were assayed with 1 mM potassium ferricyanide and 400–600 μM DCBQ. The average error in oxygen assays is approximately 10%. [b] Whole cells were assayed in BG-11 media. [c] Purification according to Noren et al. (1991); assay buffer was 1 M sucrose, 25 mM $CaCl_2$, 10 mM NaCl, and 50 mM MES–NaOH, pH 6.5. [d] 10 μM DCMU was used for inhibition studies. [e] The range of oxygen activity is 360–660 for wild type* and 280–340 for DE170D1. [f] The range of oxygen activity is 1600–2300 for wild type* and 510–640 for DE170D1. [g] Values shown are a representative set for a single purification.

treated with 20 mM hydroxylamine (from a 500 mM stock buffered with 50 mM MES–NaOH, pH 6.0) for 30 min in complete darkness. The amount of manganese that was reduced by hydroxylamine could be quantified by measurement of the six-line EPR spectrum of manganese(II) and by comparison to a manganese standard curve containing 20 mM NH_2OH (Mei & Yocum, 1991; Yocum et al., 1981). Control experiments with wild-type* membranes at the same chlorophyll concentration showed that 10, 40, and 160 mM NH_2OH gave the same amount of hydroxylamine-reducible manganese, on a chlorophyll basis, as 20 mM NH_2OH. DNase treatment of the thylakoids (Noren et al., 1991) was found to be critical in obtaining reproducible results.

Detection of Chlorophyll a Fluorescence. Chlorophyll *a* fluorescence was detected with a Walz (Effeltrich, Germany) pulse-modulation chlorophyll fluorometer [described in Schreiber (1986)] that was interfaced with a Zenith personal computer via the Walz DA-100 data acquisition system. Monitoring flashes (λ = 650 nm) of 1 μs duration were applied at a frequency of 1.6 kHz, except as noted. Single saturating actinic flashes (≈11 μs width at half-maximum) were provided by a Walz XST-103 xenon flash lamp, which incorporates a 2 mm thick Schott BG-18 blue-green filter at its exit. Multiple saturating actinic flashes (≈2 μs width at half-maximum), applied at 20 Hz, were provided by an EG&G FX-193 xenon flash lamp and were passed through a 2 mm thick Corning/Kopp CS-4-96 blue filter. The photodiode detector was protected by a 1 mm thick Schott RG-9 broad-band red filter. To prevent saturation, the photodiode amplifier was short-circuited for ≈90 μs when each actinic flash was triggered. Because the amplified signal requires ≈30 μs to restabilize, data acquisition was interrupted for a total of ≈120 μs when each actinic flash was triggered.

EPR Measurements. Room temperature EPR spectroscopy was performed at X band using a Varian E4 spectrometer that was equipped with a TE cavity and that was interfaced to a Macintosh IIcx computer through a Keitheley 195A digital multimeter and an Iotech 488A bus controller. For illumination in the cavity, a Dolan-Jenner Model 180 illuminator that was equipped with a fiber-optic light guide was used. The light was saturating for the chlorophyll concentrations that were employed. The data acquisition program was the generous gift of Prof. J. Golbeck (University of Nebraska). Data analysis, including double integration for spin quantitation, was performed using "IGOR" software (Lake Oswego, Oregon). Spectral conditions are given in the figure legends, and purified PSI was used as a *g*-value standard (Noren et al., 1991).

RESULTS

Growth Characteristics and Oxygen Evolution Activities. The DE170D1 mutant was found to be photoautotrophic and to have the same growth rate as wild-type* cells when grown in the absence of glucose. This growth rate was determined by the optical density of the cultures at 730 nm, and the doubling times were 14–15 h for both the wild-type* and DE170D1 strains. In contrast, the DN170D1 and DA170D1 mutants were found to be obligate photoheterotrophs, requiring 5 mM glucose for propagation. Table I compares oxygen evolution rates of whole cells from wild-type*, DE170D1, and DN170D1 strains. Rates of oxygen evolution in wild-type* cultures varied between 360 and 660 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}, while oxygen measurements on cells of DN170D1 and DA170D1 gave less than 50 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}. Cells of DE170D1 have oxygen evolution rates that are approximately 50% that of wild type*, although there is variation in the specific activity which we believe to reflect differences in the PSII/PSI ratio (see below). The percent inhibition of oxygen evolution by DCMU was similar in the wild-type* and DE170D1 cells, indicating that the mutation had no dramatic effect on the interaction of DCMU with the acceptor side of photosystem II.

Quantitation of Photosystem II Reaction Centers in Intact Cells. The PSII content of *Synechocystis* 6803 cells was determined on the basis of [^{14}C]DCMU binding assays. For a typical set of cultures, wild-type*, DE170D1, DN170D1, and DA170D1 cells were found to bind one DCMU per 530 ± 30, 630 ± 20, 380 ± 30, and 410 ± 40 chlorophylls, respectively. The K_D for DCMU binding was 10–12 nM for the wild-type* and DE170D1 strains and 15–18 nM for the DN170D1 and DA170D1 strains. Assuming that there is one tightly bound DCMU site per PSII reaction center, these DE170D1, DN170D1, and DA170D1 cells contained approximately 85, 140, and 130% of the PSII content of wild-type* cells, respectively, on a chlorophyll basis. However, there was variation in the PSII contents of cultures grown on different days; other cultures of DE170D1 and DN170D1 appeared to have approximately the same PSII content as wild-type* cells. Taking this variation into account, we conclude that wild-type*, DE170D1, and DN170D1 cells generally contain similar amounts of PSII on a chlorophyll basis (approximately 1 PSII per 520 chlorophylls). Lower PSII contents, giving approximately one DCMU binding site per 1000 chlorophylls, have been reported previously for *Synechocystis* 6803 (Vermaas et al., 1990; Cao et al., 1991). The approximately 2-fold increase in PSII content, on a chlorophyll basis, in our cultures is consistent with the 2-fold increase in oxygen-evolution activity that we observe when compared to these earlier reports.

Fluorescence Characteristics of Intact Cells. To determine the nature of the lesion that slows oxygen evolution in DE170D1 and essentially abolishes it in DN170D1 and DA170D1, the fluorescence properties of mutant and wild-type* cells were examined (Figures 1 and 2). The chlorophyll *a* fluorescence of cyanobacteria and chloroplasts arises primarily from PSII and is governed by the redox states of

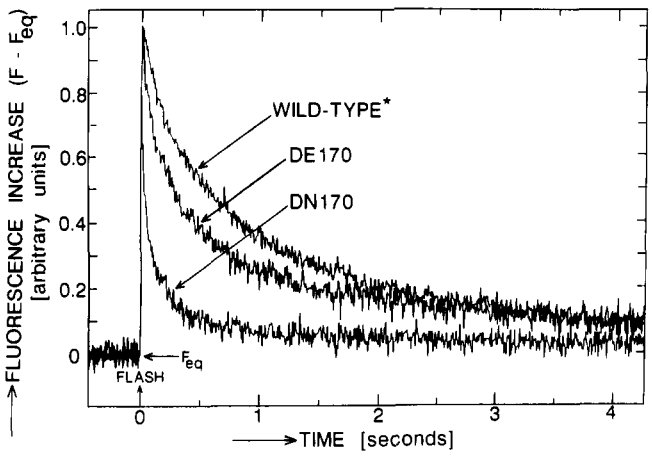

FIGURE 1: Charge recombination kinetics between Q_A^- and oxidized PSII electron donors in whole cells of *Synechocystis* sp. PCC 6803 as measured by the decay of chlorophyll *a* fluorescence following a saturating flash given in the presence of DCMU. Conditions: 20 μg of chl in 0.58 mL of 50 mM MES–NaOH, 25 mM $CaCl_2$, and 10 mM NaCl, pH 6.5, 22 °C. Samples were incubated in darkness for 10 min in the presence of 0.3 mM *p*-benzoquinone and 1 mM potassium ferricyanide before DCMU was added to a concentration of 40 μM (the final ethanol concentration was 2%). Each trace represents the computer average of four traces. The traces were normalized to their maximum ($F - F_{eq}$) values. The initial $(F - F_{eq})/F_{eq}$ values, measured ≈120 μs after the actinic flashes, were 0.42, 0.26, and 0.42 for wild-type*, DE170D1, and DN170D1 cells, respectively. Cells from DA170D1 (not shown) exhibited a halftime of 15 ± 4 ms and an initial $(F - F_{eq})/F_{eq}$ of 0.27. For the definition of F_{eq}, see Results.

Q_A (Duysens & Sweers, 1963) and P_{680} (Butler, 1972; den Haan et al., 1974) [for reviews, see van Gorkom (1986), Holzwarth (1991), and Krause and Weis (1991)]. The fluorescence of dark-adapted material, F_o, is low. Charge separation generates the state $P_{680}^+Q_A^-$; the fluorescence level of this state is similar to that of dark-adapted material because P_{680}^+ quenches chlorophyll *a* fluorescence. The reduction of P_{680}^+ by Z generates the state $P_{680}Q_A^-$; the fluorescence level of this state (F_{max}) is higher than F_o by a factor of 1.8–5. As Q_A^- is oxidized by Q_B or by the donor side of PSII, the fluorescence decreases from F_{max} to F_o. In spinach chloroplasts in the presence of DCMU, which blocks electron transfer from Q_A^- to Q_B, Q_A^- is oxidized by the S_2 state of the manganese complex with a halftime of 1.5–5 s (Lavergne & Etienne, 1980; Robinson & Crofts, 1983). However, the rate of Q_A^- oxidation by S_2 is 0.7–0.8 s in intact cells or thylakoid membranes of *Synechocystis* 6803 (Nixon & Diner, 1990; Cao et al., 1991). If the manganese complex is absent, Q_A^- is oxidized by Z^+ with a much shorter halftime, which is 80–100 ms at pH 6.5 (Dekker et al., 1984a; Metz et al., 1989). This recombination time has been found to depend markedly on pH (Dekker et al., 1984a). In cells of *Synechocystis* 6803, the fluorescence yield is believed to be directly proportional to the concentration of Q_A^- (Philbrick et al., 1991). Consequently, the decay of chlorophyll *a* fluorescence that follows a saturating light flash given in the presence of DCMU is a measure of charge recombination between Q_A^- and the donor side of PSII. Importantly, the decay of chlorophyll *a* fluorescence is sensitive to the presence or absence of a functional manganese complex.

Figure 1 shows the decay of chlorophyll *a* fluorescence in wild-type*, DE170D1, and DN170D1 cells after a saturating flash was given in the presence of DCMU. The cells were preincubated in darkness with *p*-benzoquinone to ensure that all Q_B^- was oxidized before DCMU was added (Wollman, 1978; Bowes & Crofts, 1980; Lavergne & Etienne, 1980).

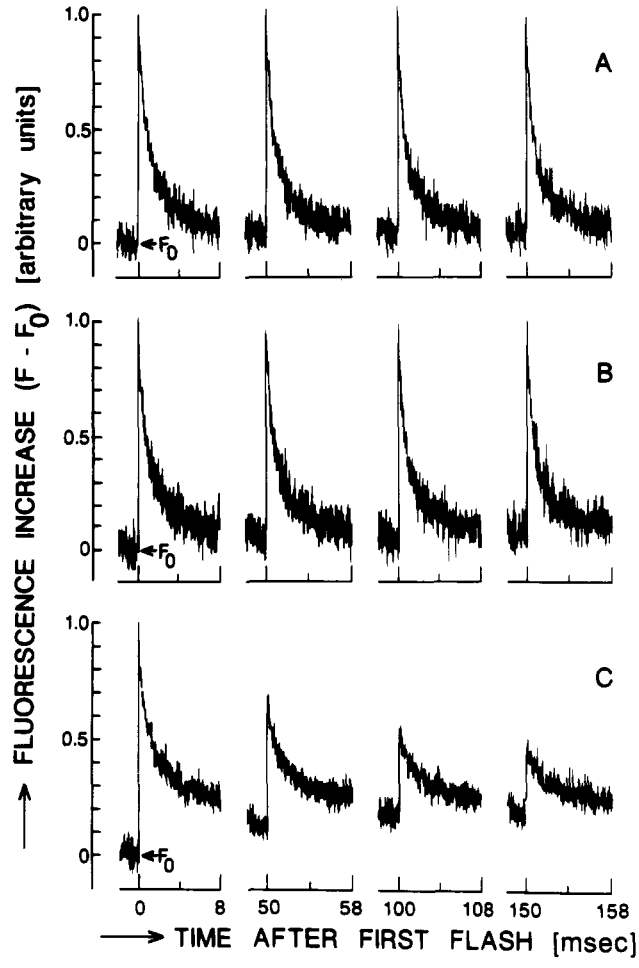

FIGURE 2: Chlorophyll *a* fluorescence produced by each of four saturating actinic flashes given at 50-ms intervals to whole cells of *Synechocystis* sp. PCC 6803. Ten milliseconds of data are shown for each flash. (A) Wild type*, (B) DE170D1, (C) DN170D1. Conditions: 20 μg of chl in 0.58 mL of 50 mM MES–NaOH, 25 mM $CaCl_2$, and 10 mM NaCl, pH 6.5, 22 °C. Samples were incubated in darkness for 10 min prior to measurement. The modulation frequency of the monitoring flashes was increased from 1.6 to 100 kHz for 10 ms, beginning 2 ms before each flash. The vertical scales of the three flash series are normalized to the maximum ($F - F_o$) values observed after the first flashes. The initial $(F - F_o)/F_o$ values, measured ≈120 μs after the first flashes, were 0.33, 0.24, and 0.39 for wild-type*, DE170D1, and DN170D1 cells, respectively. Data obtained with cells of DA170D1 (not shown) resembled the data obtained with cells of DN170D1.

Ferricyanide was included in the incubation mixture to maintain the *p*-benzoquinone in its oxidized form. The overall decay halftimes were 0.53 ± 0.03 s, 0.26 ± 0.03 s, and 34 ± 4 ms for wild-type*, DE170D1, and DN170D1 cells respectively (Figure 1) and 15 ± 4 ms for cells of DA170D1 (not shown). The rapid decays observed for DN170D1 and DA170D1 are consistent with charge recombination taking place between Q_A^- and Z^+ in these mutants, while the considerably slower decay observed in DE170D1 is consistent with charge recombination taking place between Q_A^- and the manganese cluster in this mutant.

The decay halftimes that we observe for wild-type* and DE170D1 cells are somewhat faster than those previously reported for *Synechocystis* 6803 using unmodulated fluorescence detection (Nixon & Diner, 1990, 1992). With unmodulated detection, the small fraction of centers that are excited by the monitoring flashes is able to recover before the next monitoring flash is applied. However, with the Walz fluorometer, there is insufficient time between the monitoring flashes for all of the excited centers to recover. These flashes

are applied at 1.6 kHz, and on this timescale, charge recombination between Q_A^- and the donor side of PSII is a slow process. Because a fraction of those centers that are excited by the monitoring flash will not recover before the next monitoring flash is applied, a steady-state level of excited centers, having Q_A reduced, is established. As a result, the observed fluorescence increases rapidly from F_o to a steady-state level, F_{eq}, when the monitoring flashes are turned on. Under the conditions employed in this study, F_{eq} is approximately 1.2 F_o. PSII appears to be highly heterogeneous with respect to its kinetics of charge recombination (Lavergne & Etienne, 1980; Robinson & Crofts, 1983; Nixon & Diner, 1990, 1992; Cao et al., 1991). More of the PSII centers with long charge recombination halftimes will have Q_A reduced throughout the time between the monitoring flashes and, therefore, will contribute to F_{eq}. Consequently, more PSII centers having *shorter* halftimes will undergo charge separation, generating Q_A^-, during the actinic flash and will contribute to the observed charge recombination kinetics.

An alternative assay for the presence or absence of a functional manganese complex is to observe the maximal level of fluorescence following each of a series of closely spaced saturating actinic flashes given in the absence of DCMU (Nixon & Diner, 1990, 1992). The highly fluorescent state $Z^+P_{680}Q_A^-Q_B$ is formed 40–280 ns after the first flash (Bret-tel et al., 1984). The subsequent fluorescence decay represents the oxidation of Q_A^- by Q_B. The manganese complex normally reduces Z^+ in 30–1300 μs (Dekker et al., 1984b). However, if the manganese complex is absent or nonfunctional, Z^+ will be reduced by Q_B^- or by an alternate donor at a slower rate. If a second saturating flash is given before Z^+ has been reduced, then the low-fluorescent-state $Z^+P_{680}^+Q_A^-Q_B^-$ will be formed. Consequently, in the absence of a functional manganese complex, the maximal fluorescence that follows the second and subsequent flashes will be substantially less than that which follows the first flash. As demonstrated in Figure 2, the maximal fluorescence of both wild-type* and DE170D1 cells is essentially the same following each of a series of flashes given 50 ms apart. These data show that Z^+ is rapidly reduced in these cells after each flash. In contrast, the maximal fluorescence of DN170D1 cells (Figure 2) and DA170D1 cells (not shown) following the second and subsequent flashes is substantially quenched, demonstrating that the reduction of Z^+ in these mutants has been slowed considerably, from a halftime of 30–1300 μs (Dekker et al., 1984b) to halftimes of 30–40 ms. These data support the conclusions drawn from Figure 1; DE170D1 cells contain functional manganese complexes able to rapidly reduce Z^+, while DN170D1 and DA170D1 cells do not.

Purification of Photosystem II Particles. In order to further characterize the properties of the DE170D1 and DN170D1 mutants, photosystem II particles were isolated by the method of Noren et al. (1991) from mutant and wild-type* strains. In this purification method, cells are broken with glass beads, producing "thylakoid membranes" which are then solubilized by the nonionic detergent, lauryl maltoside. Solubilization is followed by two anion-exchange chromatography steps. This first column is a "Fast-flow Q" column run at pH 6.0; the second column which yields purified "photosystem II particles", is an FPLC Mono Q column run at pH 6.5. Previous characterization of this photosystem II preparation has shown that the increase in the specific activity for oxygen evolution (on a chlorophyll basis) correlates with the decrease in photosystem I contamination, indicating that the majority of centers are active in water oxidation (Noren et al., 1991).

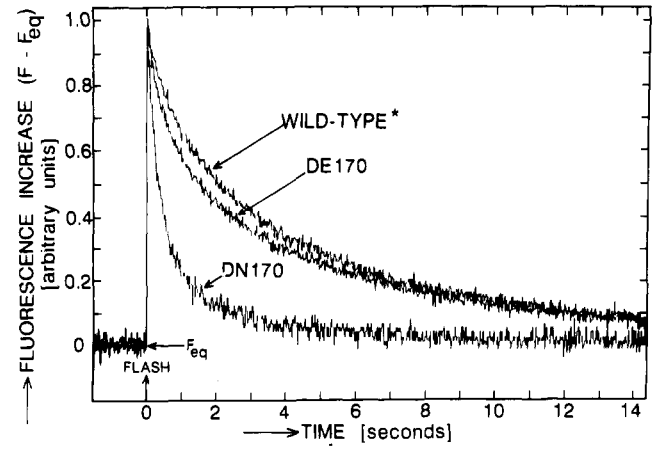

FIGURE 3: Charge recombination kinetics between Q_A^- and oxidized PSII electron donors in PSII particles from *Synechocystis* sp. PCC 6803 as measured by the decay of chlorophyll *a* fluorescence following a saturating flash given in the presence of DCMU. Conditions: 5 μg of chl in 0.58 mL of 50 mM MES–NaOH, 25 mM $CaCl_2$, 10 mM NaCl, and 1 M sucrose, pH 6.5, 4°C. Samples were incubated in darkness for 10 min in the presence of 10 μM DCBQ before DCMU was added to a concentration of 100 μM (the final ethanol concentration was 2%). The trace for DN170D1 represents the computer average of 16 traces. The traces were normalized to their maximum $(F-F_{eq})$ values. The initial $(F-F_{eq})/F_{eq}$ values, measured ≈120 μs after the actinic flashes, were 0.45, 0.51, and 0.17 for wild-type*, DE170D1, and DN170D1 particles, respectively.

Table I summarizes oxygen-evolution rates in the thylakoid membranes and photosystem II particles from all three strains. The specific activity of oxygen evolution increases by a factor of 4 when thylakoid membranes and photosystem II particles of wild type* are compared. Specific activity of oxygen evolution also increases by a factor of 2 in DE170D1. Thylakoid membranes and photosystem II particles of DN170D1 gave no detectable oxygen evolution. Table I also shows the percent of oxygen-evolution activity that is inhibited by DCMU in DE170D1 and wild-type* thylakoid membranes and photosystem II preparations. The percent DCMU inhibition is comparable in all of these samples, indicating that the purification process has not drastically affected the binding site for the inhibitor on the acceptor side of photosystem II.

Charge Recombination Kinetics in Photosystem II Particles. In Figure 3, charge recombination was measured in wild-type*, DE170D1, and DN170D1 photosystem II particles by the decay of chlorophyll *a* fluorescence after a saturating flash given in the presence of DCMU. To ensure that the quinone electron acceptors were fully oxidized, samples were incubated with 10 μM DCBQ for 10 min in darkness prior to the addition of DCMU. Benzoquinone was not employed for this purpose because, in isolated PSII particles, *p*-benzoquinone has been shown to oxidize the non-heme Fe^{2+} ion (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987). DCBQ is much less effective in promoting this oxidation of the Fe^{2+} ion (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987). The overall decay halftimes were 2.1 ± 0.1, 1.4 ± 0.1, and 0.33 ± 0.04 s for the wild-type*, DE170D1, and DN170D1 particles, respectively (Figure 3). The slow decay kinetics observed for the DE170D1 particles demonstrate that, in the vast majority of these particles, Q_A^- recombines with the S_2 state of the manganese complex. The faster charge recombination kinetics observed in the DN170D1 particles are consistent with charge recombination between Q_A^- and Z^+, as expected based on fluorescence data obtained from whole cells (Figure 1). Also, the charge recombination halftime in the DN170D1 particles decreased 4-fold as the pH was increased from pH 5.5 to pH 7.5 (data not shown); a similar

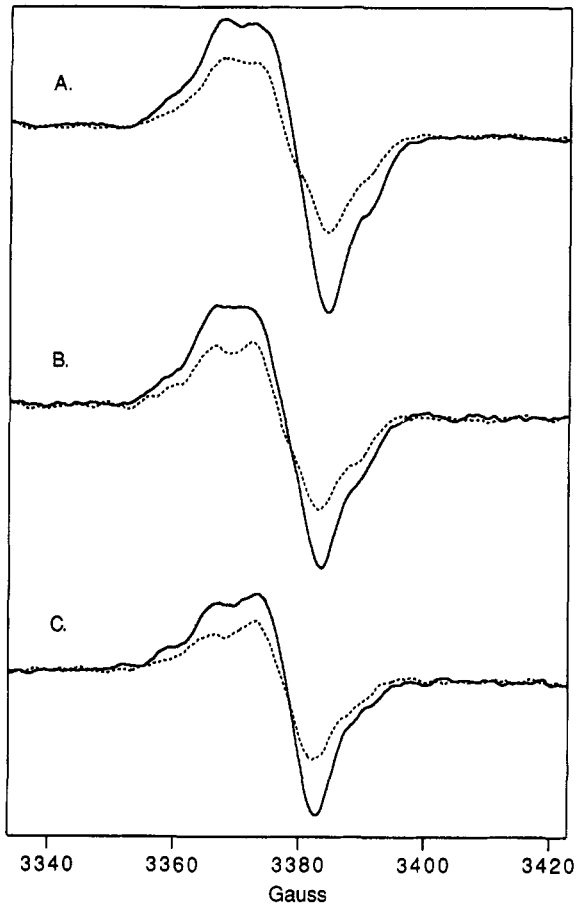

FIGURE 4: Room temperature EPR spectra of Tris-washed PSII particles from (A) wild type*, (B) DE170D1, and (C) DN170D1. Spectra taken in the light are indicated by a solid line; spectra taken in the dark are indicated by a dotted line. The chlorophyll concentrations were 0.52, 0.47, and 0.40 mg/mL for wild type*, DE170D1, and DN170D1, respectively. 1 mM ferricyanide was used as an acceptor. Spectral conditions were microwave power, 3.5 mW; field modulation, 3.2 G; time constant, 2 s; scan time, 4 min; and gain, 2.0×10^4. The *g*-value of the tyrosine radical, D^+, is known to be 2.0046 (Miller & Brudvig, 1991). For plotting, the spectra were scaled to an effective chlorophyll concentration of 0.48 mg/mL for comparison.

decrease, but of greater magnitude, has been reported for charge recombination between Q_A^- and Z^+ in PSII particles from spinach (Dekker et al., 1984a). The 4–8-fold difference in fluorescence decay halftimes observed between intact cells and photosystem II particles (cf., Figures 1 and 3) is not understood but may be due to the presence of a membrane potential in intact cells [for a review, see Witt (1979)].

Room Temperature EPR Spectra of Photosystem II Particles. The two redox-active tyrosine radicals, D^+ and Z^+, can be detected by room temperature EPR studies of wild-type*, DE170D1, and DN170D1 photosystem II particles. In Figure 4 we present room temperature EPR spectra that were recorded on Tris-washed particles. Tris washing removes manganese and the 33-kDa extrinsic protein (Cheniae & Martin, 1970; Yamamoto et al., 1981). In each panel, the dashed curve shows the EPR spectrum recorded in the dark following illumination; the signal reflects the EPR line shape of the dark-stable tyrosine radical, D^+. Spin quantitation and normalization to the wild type* shows that, typically, the D^+ contents for the mutants fall in the range of 1.0–0.9 D^+ spin per 2 cytochrome b_{559}. We estimate the error in D^+ spin quantitation as approximately 15%. In each panel, the solid curve shows the spectrum recorded in the light; the increase is caused by the generation of Z^+ in centers where

Table II: Characterization of Wild-Type* and Mutant Photosystem II Particles

	wild type*	DE170D1	DN170D1
chl/2cyt b_{559}[a] (dithionite red.)	63	68	79
chl/2cyt b_{559}[a] (ascorbate red.)	85	78	132
chl/P_{700}+[b]	660	870	400
Mn/2cyt b_{559}[c] (dithionite red.)	4.0	3.3	1.4
	(4.2–3.8)[d]	(3.6–3.1)[d]	(1.7–1.2)[d]
Mn/D^+	4.0/4.0[e]	3.3/4.0	1.8/4.0
	(4.1–3.8)[d]	(3.6–3.1)[d]	(2.1–1.5)[d]

[a] Mol/2 mol (±3%). [b] Mol/mol (± 10%). [c] Mol/2 mol. [d] Range. [e] Average normalized to 4.0.

the reduction of the tyrosine radical has been purposely interrupted by manganese removal. The increase in the amplitude of the signal is approximately 60–70% for all of the samples [(light – dark)/dark] (see below). The line shape of Z^+ in wild-type* and DE170D1 particles is similar (Figure 4A and B); we attribute the small line shape difference that we observe for the DN170D1 preparation to increased P_{700}^+ contamination in this sample (Table II). However, we cannot rule out a small change in line shape for the tyrosine radicals in the DN170D1 mutant preparation. These data show that Z^+ can be generated in wild type* and in both mutant strains.

The light-induced increase in the amplitude of the tyrosine radical is less than 100% in the Tris-washed samples characterized here. We routinely observe that Tris-washed *Synechocystis* photosystem II particles in high glycerol buffer exhibit the Z^+ EPR signal in only 60–70% of the centers, in spite of the fact that Tris treatment completely inactivates oxygen evolution (data not shown). This effect seems to be due to the high concentrations of glycerol that are necessary in order to stabilize the manganese cluster in *Synechocystis* PSII particles. In fact, we have also observed that Tris-washed spinach photosystem II membranes (Berthold et al., 1981) in glycerol buffer show a smaller light-induced increase in the tyrosine signal as compared to samples in sucrose buffer (data not shown). The origin of this effect is still under investigation, but could arise from a protein conformational change that alters the rate of charge recombination between Z^+ and Q_A^-.

In Figure 5, we present room temperature EPR spectra on wild-type* and mutant photosystem II samples that are untreated, i.e., not Tris-washed. Again, the dashed line in each panel shows the EPR line shape of D^+. Spin quantitation and normalization to wild-type* D^+ in the Tris-washed sample (Figure 4) shows that, typically, the D^+ content in these samples ranges between 1.0 and 0.9 spins per 2 cytochrome b_{559}. In the presence of a catalytically competent manganese cluster, reduction of Z^+ is rapid enough to prevent its accumulation in the light. This is illustrated in Figure 5A, which shows that in our active wild-type* particles the increase in the amplitude of the tyrosine signal upon illumination is less than 20% [(light – dark)/dark]. We attribute this small increase to Z^+ photoaccumulation in centers that lack manganese. The light-induced increase in the EPR signal is less than 40% in the DE170D1 preparation (Figure 5B) and approximately 60% in the DN170D1 preparation (Figure 5C). Note that in the DN170D1 preparation, the full amplitude of Z^+ is obtained without Tris washing (Figures 4C and 5C), supporting the fluorescence studies (Figures 1–3) where Z^+ was shown to be the terminal electron donor in these particles. However, in the DE170D1 particles, removal of manganese and the 33-kDa protein is required to observe the full complement of Z^+, consistent with the fluorescence data (Figures 1–3) showing that the manganese cluster is able to reduce Z^+ in this mutant.

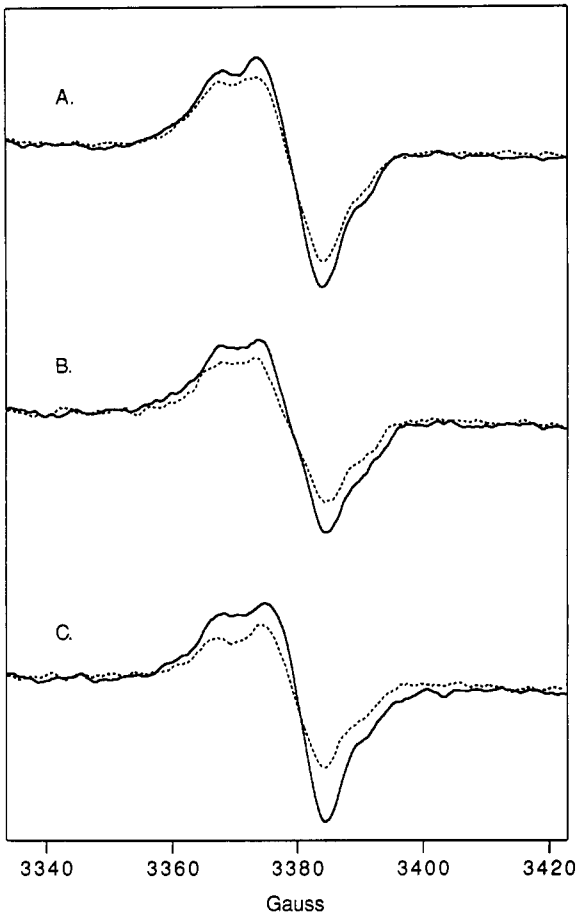

FIGURE 5: Room temperature EPR of untreated photosystem II particles from (A) wild type*, (B) DE170D1, and (C) DN170D1. Spectra taken in the light are indicated by a solid line; spectra taken in the dark are indicated by a dotted line. The chlorophyll concentrations were 0.50, 0.40, and 0.44 mg/mL for wild type*, DE170D1, and DN170D1, respectively. The spectral conditions were the same as described in Figure 4, except the gain was 4.0×10^4. The g-value of the tyrosine radical, D^+, is known to be 2.0046 (Miller & Brudvig, 1991). For plotting, data were scaled to 0.48 mg of chl/mL and then by a factor of 2 to account for the difference in gain between Figures 4 and 5. Therefore, amplitudes in Figures 4 and 5 are directly comparable.

Quantitation of Manganese. The fluorescence and EPR studies that are described above show that the rate of electron transfer between the manganese cluster and tyrosine Z^+ is dramatically slowed in the DN170D1 mutant. If aspartate 170 is involved in stabilization or assembly of the manganese cluster, then we expect that the DN170D1 mutation should also decrease PSII manganese content. Accordingly, we determined the amount of photosystem II manganese found in thylakoid membranes, in partially purified samples after the Fast-flow Q column, and in photosystem II particles from wild-type*, DE170D1, and DN170D1 strains.

Tables II and III show quantitation of specifically bound manganese in wild-type*, DE170D1, and DN170D1 photosystem II particles. Total manganese was released with hydrochloric acid by the methodology described by Yocum et al. (1981) and Noren et al. (1991). This manganese is likely to represent only tightly bound manganese because we detect no manganese(II) hexaquo six-line signals in the EPR spectra of these particles before the addition of HCl, because ion-exchange chromatography is used in the purification and because high concentrations of calcium are always present during the chromatography steps. Excess calcium has been shown to displace adventitious manganese (Yocum et al., 1981).

Table III: Manganese Quantitiation in Wild-Type* and Mutant Strains[a]

	wild type*	DE170D1	DN170D1
PSII particles	64 (60–66)[b]	83 (76–89)[b]	220 (190–260)[b]
Fast-flow Q samples	120 (110–140)[b]	140 (120–160)[b]	590 (530–680)[b]
thylakoid membranes (HCl-released)	90 (70–110)[b]	80 (70–100)[b]	110 (100–120)[b]
thylakoid membranes (hydroxylamine-reduced)	440 (430–450)[b]	530 (520–540)[b]	≥2700

[a] Mol of chlorophyll/4 mol of manganese. [b] Range.

The presence of varying amounts of photosystem I in the three preparations necessitates the normalization of manganese content to photosystem II reaction center size in each preparation. Previous analysis of this type of preparation has indicated that there are approximately 2 dithionite-reducible cytochrome b_{559}, 4 manganese, and 1 D^+ spin per 60 chlorophylls (Noren & Barry, 1992). Therefore, an optical absorption difference assay that determines the number of chlorophylls per 2 mol of cytochrome b_{559} was used to determine reaction center size. We have determined the amount of cytochrome b_{559} using both ascorbate and dithionite as the reductants (Table II). Cytochrome b_{559} quantitation shows that photosystem II particles from wild-type* and DE170D1 strains have reaction center sizes of approximately 65 chlorophylls, while DN170D1 has a slightly larger reaction center size (Table II). Optical P_{700}^+ quantitation (Table II) shows that the larger reaction center size of the DN170D1 preparation is due to increased photosystem I contamination. Estimating an intrinsic reaction center size of 60 for photosystem II and 85 for photosystem I (Noren & Barry, 1992), we estimate that the DN170D1 preparation contains 5 PSII/1 PSI.

Table III gives the number of chlorophylls per 4 manganese in each photosystem II preparation. Using the determinations of reaction center size described above, we normalize these values in Table II. We find 4.0 manganese per 2 cytochrome b_{559} in the wild-type* preparation. The DE170D1 preparation contains 3.3 manganese per 2 cytochrome b_{559}, while the DN170D1 preparation contains an average of 1.4 manganese per 2 cytochrome b_{559} (Table II).

We also normalize the amount of manganese found in DE170D1 and DN170D1 particles to the amount in wild type* through spin quantitation of the room temperature D^+ EPR signal, measured in the same samples. The EPR data on these particular samples were not corrected with a spin standard.[2] Instead, we arbitrarily set the manganese content in wild type* to 4.0 manganese per D^+ for comparison to the b_{559} data. We find 3.3 manganese per D^+ in DE170D1, and 1.8 manganese per D^+ in DN170D1 particles (Table II). In other words, correction by either dithionite-reducible cytochrome b_{559} or by quantitation of the D^+ signal indicates that manganese content is reduced by 20% in DE170D1 reaction centers and by approximately 60% in DN170D1 reaction centers.

To show that the low content of manganese in the DN170D1 preparation is not the result of our preparative method, we determined the manganese content earlier in the purification procedure, after the first of the two anion-exchange columns, the Fast-flow Q column. We expect the content of adventitiously bound manganese in this material to be low because

[2] On a different, but representative, wild-type* sample, we found 51 chl/D^+ through the use of a spin standard [see Noren and Barry (1992)].

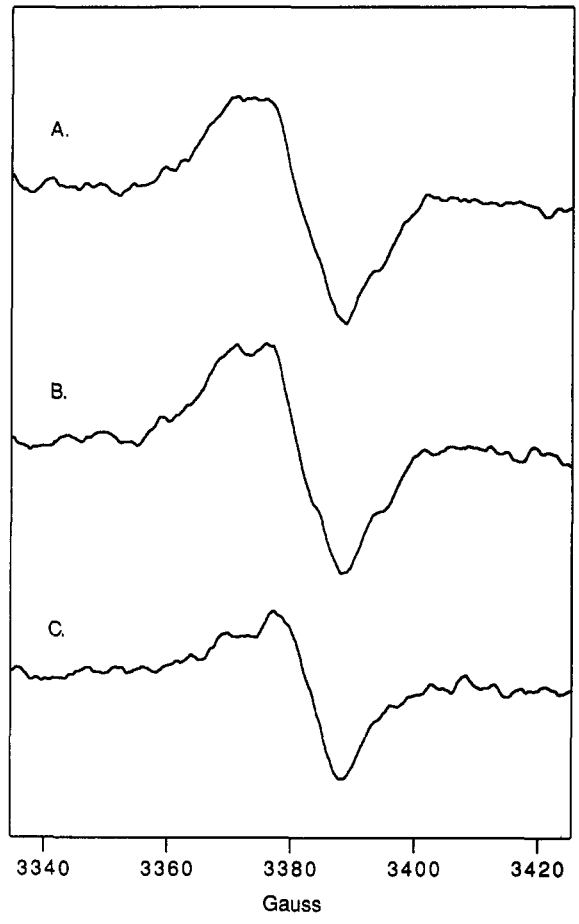

FIGURE 6: Room temperature EPR of samples after the Fast-flow Q column from (A) wild type*, (B) DE170D1, and (C) DN170D1. Spectra were taken in the dark, and 0.5 mM ferricyanide and 0.5 mM ferrocyanide were used as acceptors. The chlorophyll concentrations were 0.65, 0.71, and 0.57 mg/mL for wild type*, DE170D1, and DN170D1, respectively. The gain was 2.5×10^4 for wild type* and DE170D1 and 4.0×10^4 for DN170D1. The *g*-value of the tyrosine radical, D^+, is known to be 2.0046 (Miller & Brudvig, 1991). For plotting, the data were scaled to 0.65 mg of chl/mL and a gain of 2.5×10^4. All other conditions were as described in Figure 4.

it has been chromatographed on one ion-exchange column in the presence of high concentrations of calcium. Release of total manganese with HCl gives 4 manganese per 120 chlorophylls in wild type*, 4 manganese per 140 chlorophylls in DE170D1, and 4 manganese per 590 chlorophylls in DN170D1 samples (Table III). Again, we must correct the manganese content by the photosystem II reaction center size. Contamination from other cytochromes precludes the use of cytochrome b_{559} as a measure of reaction center size at this early state of the purification procedure (Noren et al., 1991). However, we can correct for variation in reaction center size by use of the D^+ spin content, measured in the same samples. The room temperature EPR spectra of D^+ used for this correction are shown in Figure 6. Note that, because of the large amount of photosystem I contamination at this point in the purification procedure, we cannot observe the D^+ signal in the presence of potassium ferricyanide, as we can in the final purified photosystem II particles (Figures 4 and 5). Instead, the data in Figure 6 were recorded in the dark in the presence of an equimolar mixture of ferricyanide and ferrocyanide to keep the P_{700}^+ signal reduced. The EPR spectra of D^+ in these samples are similar to the D^+ spectra in photosystem II particles (Figures 4 and 5). For comparison with manganese quantitation in photosystem II particles, we arbitrarily normalized the wild-type* manganese content to 4.0 manganese per D^+. Correction of manganese content by D^+ spin content in the mutants gives 3.8 and 1.2 manganese per D^+ for DE170D1 and DN170D1, respectively. These data indicate that, at this point in the purification, manganese content is reduced by approximately 70% in DN170D1 reaction centers, compared to wild type*. This result is in reasonable agreement with the determination described above for the final purified photosystem II particles (Table II). These data indicate that manganese is not gradually lost from the DN170D1 preparation during the purification procedure.

Finally, we determined the photosystem II manganese content in thylakoid membranes of wild-type* and mutant strains (Table III). Quantitation of manganese in the thylakoid membranes is complicated by the large amount of excess manganese that is released by HCl treatment of this material. EDTA washing, either in the presence or in the absence of the ionophore A23187 does not significantly lower the amount of this adventitiously bound manganese. Wild-type*, DE170D1, and DN170D1 membranes contained approximately the same amount of HCl-releasable manganese (Table III). In an attempt to observe only the manganese that is associated with photosystem II, membranes were treated with hydroxylamine. Such treatment will reduce photosystem II manganese, producing a manganese(II) six-line EPR signal (Mei & Yocum, 1991; Yocum et al., 1981). The amount of manganese released can be determined by comparison with a standard curve. Previous studies of hydroxylamine reduction of photosystem II manganese have shown that hydroxylamine reduces 3–4 manganese per photosystem II reaction center (Yocum et al., 1981; Ghanotakis et al., 1984; Tamura & Cheniae, 1985).

Before the addition of hydroxylamine, there was either no detectable or a very small amount [corresponding to $<1.2\ \mu M$ Mn(II)] of six-line manganese(II) EPR signal in these samples. When there was a detectable manganese signal before hydroxylamine addition, the metal concentration was determined by using the appropriate manganese standard curve, and the amount of hydroxylamine reducible manganese was corrected by this amount. Quantitation of the six-line EPR signal, produced after hydroxylamine addition, showed 4 manganese per approximately 440 chlorophylls in wild-type* membranes and 4 manganese per 530 chlorophylls in DE170D1 membranes. The amount of hydroxylamine-reducible manganese in DN170D1 membranes was near our detection limit, giving $\geq$2700 chlorophylls per 4 manganese (Table III). Since the PSII content, on a chlorophyll basis, is similar in wild type*, DE170D1, and DN170D1 (see above) and since the amount of hydroxylamine-reducible manganese is at least 80% lower in DN170D1 than in wild-type* or DE170D1 membranes, we conclude that *thylakoid membranes* from DN170D1 are deficient in specifically bound PSII manganese. In making this conclusion, we must assume that the aspartate to asparagine mutation does not change the accessibility of the metal cluster to the reductant.

In summary, manganese quantitation shows that PSII manganese content is decreased substantially in PSII particles, Fast-flow Q samples, and thylakoid membranes from DN170D1, but is not greatly affected in DE170D1 samples.

Content of the 33-kDa Extrinsic Protein. Figure 7 shows that the decrease in manganese content in DN170D1 photosystem II particles is accompanied by a decrease in the amount of the 33-kDa extrinsic protein. Figure 7 is a Western blot developed with an antibody against the spinach 33-kDa extrinsic protein; this antibody cross-reacts with a single band in *Synechocystis* 6803 thylakoid membranes and particles

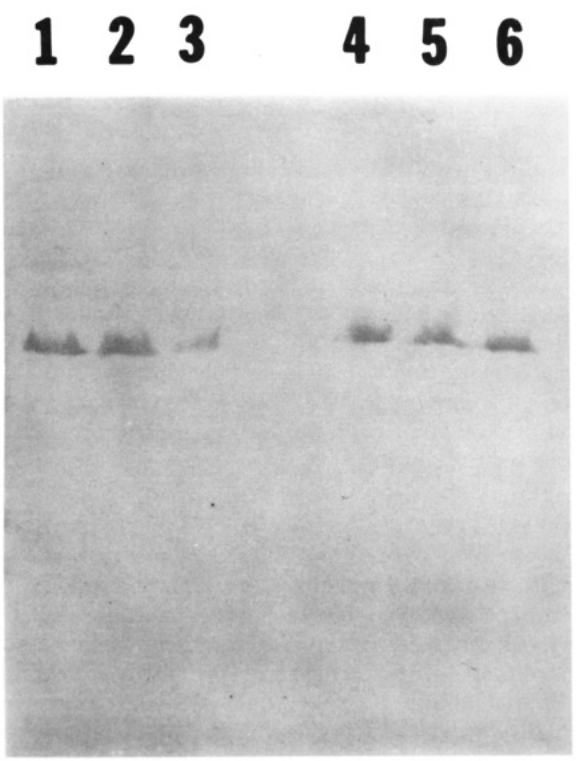

FIGURE 7: Western blot of (1) wild-type*, (2) DE170D1, (3) DN170D1 photosystem II particles and of (4) wild-type*, (5) DE170D1, and (6) DN170D1 thylakoid membranes. For thylakoid membranes, 4 μg of chl were loaded; for photosystem II particles, equal amounts of reaction centers were loaded (35.5 pmol) based on cytochrome b_{559} quantitation to determine reaction center size. The blot was probed with a rabbit polyclonal antibody that was raised against the purified spinach 33-kDa protein (Noren et al., 1991).

(Noren et al., 1991). Approximately *equal* amounts of the 33-kDa protein are detected in wild-type*, DE170D1, and DN170D1 thylakoid membranes (lanes 4–6). Photosystem II particles of wild type* and DE170D1 contain comparable amounts of the 33-kDa extrinsic protein when compared on a reaction center basis, while DN170D1 particles lose the majority of the 33-kDa protein during purification (lanes 1–3). Use of an antibody against the D2 polypeptide gave an approximately equal reaction in each of lanes 1–3 and of lanes 4–6 (data not shown).

DISCUSSION

We have used a highly active photosystem II preparation to characterize mutations constructed at residue 170 of the D1 polypeptide in photosystem II. We conclude that the carboxylate found at residue 170 influences the stability or assembly of the manganese cluster. First, elimination of this carboxylate by the introduction of an asparagine residue results in a non-photoautotrophic (PS–) phenotype. EPR measurements on purified PSII particles demonstrate that the block in steady-state oxygen evolution does not involve electron transfer between Z and Q_A since Z^+ can be photoaccumulated in Tris-washed mutant samples. Chlorophyll *a* fluorescence was used to monitor charge recombination times in both DN170D1 cells *and* DN170D1 PSII particles; these data show an inhibition or dramatic slowing of electron transfer from manganese to Z^+. Measurement of the maximal level of fluorescence following each of a series of saturating actinic flashes also leads to the conclusion that manganese is not able to rapidly reduce Z^+ in DN170D1 cells since the maximal fluorescence is quenched following the second and subsequent flashes. Steady-state EPR measurements on untreated DN170D1 PSII samples support the results from charge recombination experiments, since these spectra show a defect in the reduction of Z^+. Therefore, we conclude that tyrosine Z is the terminal electron donor in the reaction center of DN170D1.

Complete inhibition of electron transfer or a dramatic decrease in electron-transfer rate between manganese and Z^+ could potentially be caused by several effects, including a change in redox potential of the manganese cluster, a dramatic change in the characteristics of the electron-transfer pathway, and/or a loss of the manganese cluster. We expect that, if Asp-170 influences the assembly or stability of the manganese cluster, the third possible explanation will hold and the metal content of the DN170D1 mutant will be diminished. Indeed, manganese quantitation shows a 70% decrease in the amount of tightly bound manganese per reaction center in DN170D1 samples after the Fast-flow Q column and a 60% decrease in manganese per reaction center in DN170D1 photosystem II particles, compared to wild type*. Furthermore, we also observe a substantial decrease in the content of hydroxylamine-reducible manganese in DN170D1 thylakoid membranes. These results suggest that the affinity for PSII manganese is decreased in the DN170D1 mutant and that loss of manganese leads to the alterations in electron-transfer properties that are documented here. Note that there is residual manganese bound in PSII particles from this mutant; however, fluorescence and EPR assays show that this manganese does not rapidly reduce Z^+.

Additional evidence that Asp-170 influences the assembly or stability of the manganese cluster comes from analysis of the DE170D1 mutant. Substitution of a glutamate residue for aspartate results in a functional metal center in spite of the increase in size of the side chain. Measurement of the maximal fluorescence that follows each of a series of saturating actinic flashes demonstrates that Z^+ can be rapidly rereduced in DE170D1, indicating that the manganese cluster is functioning as the terminal electron donor. However, the properties of the manganese cluster are altered. For example, we attribute the kinetics of fluorescence decay in both cells and purified photosystem II particles to charge recombination between Q_A^- and the S_2 state of the manganese complex. However, the recombination kinetics of DE170D1 are considerably more heterogeneous than those of wild type*, having both faster and slower components. Furthermore, the overall halftimes are about 2-fold lower than those observed in wild-type* cells and particles. These data suggest that the manganese cluster in DE170D1 may have an altered redox potential. Evidence for heterogeneity in the functional properties of the manganese cluster also comes from room temperature EPR studies of photosystem II particles. EPR analysis of DE170D1 shows that Z^+ can be photoaccumulated in Tris-washed material, but not all of the centers are capable of steady-state reduction of Z^+ in untreated, non-Tris-washed material. Also, the affinity for manganese in DE170D1 may be slightly diminished since purified DE170D1 photosystem II particles contain 20% less manganese than wild-type* particles.

The results presented here argue that the majority of the DE170D1 centers contain a functional but perturbed manganese cluster. This perturbation is also reflected in the specific activity for oxygen evolution. Rates of oxygen evolution in DE170D1 cells are decreased by approximately a factor of 2, when compared to wild-type* cells. This lower rate in whole cells is not due to decreased photosystem II content, since [^{14}C]DCMU binding studies demonstrate that wild-type* and

DE170D1 cells contain similar amounts of PSII on a chlorophyll basis. Moreover, oxygen evolution rates for the DE170D1 PSII particles are only one-third of wild-type* rates, after correction for loss of manganese from 20% of the DE170D1 centers. We can account for the low rate of oxygen evolution in the DE170D1 mutant in two ways. In one explanation, all of the DE170D1 centers have an assembled manganese cluster, but the turnover rate of the S states is decreased by a factor of 2–3. Such a decrease in catalytic efficiency could be caused by an alteration in the metal cluster that is induced by the mutation. The change in the lifetime of the S_2 state, which has been described in an independently constructed DE170D1 mutant, may be consistent with this proposal (Nixon & Diner, 1992). A second explanation for the lower activity of DE170D1 is that PSII reaction centers of this mutant contain fewer functional clusters, perhaps because the mutation decreases the probability of correctly assembling the cluster (Nixon & Diner, 1992). However, we consider this explanation less likely than the first because manganese quantitation indicates that this mutant retains 80% of its tightly bound metal throughout the purification procedure and because our fluorescence data suggest that rapid reduction of Z^+ from manganese takes place in the majority of DE170D1 reaction centers.

We find it intriguing that loss of manganese in the DN170D1 mutant is correlated with loss of the 33-kDa protein in the purified reaction center. We suggest that the aspartate to asparagine mutation, which decreases manganese binding affinity, also decreases the binding affinity of the 33-kDa protein, allowing the extrinsic protein to be removed from the reaction center complex during anion-exchange chromatography. This result may be an indication that manganese directly or indirectly mediates the binding of the 33-kDa protein to the reaction center. Studies of photosystem II membranes from spinach and of photosystem II particles from the YF161D1 mutant of *Synechocystis* 6803 have also suggested that loss of manganese affects 33-kDa protein binding (Ghanotakis et al., 1984; Miyao & Murata, 1989; Kavelaki & Ghanotakis, 1991; Noren & Barry, 1992).

Our conclusion that loss of manganese precedes loss of the 33-kDa protein in the DN170D1 mutant, and not the contrary, rests on the following observations. First, it is unlikely that the primary lesion in this mutant is loss of the 33-kDa protein, because cyanobacteria, in which the gene for the 33-kDa protein has been deleted or inactivated by an insertion (Bockholt et al., 1991; Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991), are still photoautotrophic. In contrast, DN170D1 cells are obligate photoheterotrophs. Second, DN170D1 membranes are already deficient in their content of hydroxylamine-reducible manganese when compared to wild type*, although the full complement of the 33-kDa protein is observed in the DN170D1 membranes. We propose, therefore, that manganese loss in the DN170D1 mutant destabilizes the binding of the 33-kDa protein.

In summary, we conclude that aspartate 170 influences the assembly or stability of the manganese cluster. Nixon and Diner (1992) have come to the same conclusion through a study of *Synechocystis* mutants and mutant photosystem II preparations that contain no functional manganese. Substitution of asparagine for aspartate at position 170 decreases the amount of bound manganese and dramatically affects the rate of electron transfer from manganese to tyrosine Z, although the change in the size of the side chain is small. Substitution of glutamate at this site also perturbs the properties of the manganese cluster, showing that the perturbation in the DN170D1 mutant is not simply due to the loss of a negative charge. While we cannot rule out the possibility that the DE170D1 and DN170D1 mutations have induced conformational changes in the reaction center that indirectly cause the effects described here, our results, which include a dramatic decrease in the content of bound metal and a loss of enzymatic activity, are typical of those reported in other systems when metal ligands are altered (Haiech et al., 1991; Makaroff et al., 1986; Nakai et al., 1990; Rothery & Weiner, 1991). Therefore, our results are consistent with the possibility that aspartate 170 directly coordinates either manganese or a calcium ion that is an intrinsic component of the water-oxidizing site and is necessary for the structural integrity of the cluster. Definitive evidence regarding the amino acid residues that ligate manganese awaits further spectroscopic analysis.

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