Amino Acid Residues That Influence the Binding of Manganese or Calcium to Photosystem II. 1. The Lumenal Interhelical Domains of the D1 Polypeptide†

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ABSTRACT: To identify amino acid residues that ligate the manganese and calcium ions of photosystem II or that are otherwise crucial to water oxidation, site-directed mutations were constructed in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 at all conserved carboxylate, histidine, and tyrosine residues in the lumenal interhelical domains of the D1 polypeptide. Mutants with impaired photoautotrophic growth or oxygen evolution were characterized *in vivo* by measuring changes in the yield of variable chlorophyll *a* fluorescence after a saturating flash or brief illumination given in the presence of an electron-transfer inhibitor or following each in a series of saturating flashes given in the absence of inhibitor [Chu, H.-A., Nguyen, A. P., & Debus, R. J. (1994) *Biochemistry 33,* 6137−6149]. Mutants were also characterized after propagation in media having other cations substituted for calcium. We conclude that Asp-59 and Asp-61 may ligate calcium, that Asp-59, Asp-61, Glu-65, and His-92 influence the properties of the manganese cluster without significantly affecting its stability or ability to assemble, that Glu-189 plays an important structural role in maintaining the catalytic efficiency of the Mn cluster and partly influences the cluster's stability or ability to assemble, that His-92 and Glu-189 influence the binding of calcium, and that His-190 strongly influences the redox properties of the secondary electron donor, Y_Z^{ox}, and either ligates manganese or serves as a crucial base or hydrogen bond donor. In addition, we conclude that Asp-170 may ligate manganese, but that its replacement with Val, Leu, or Ile causes structural perturbations that partly compensate for the loss of the carboxylate moiety.

The oxygen-evolving complex of photosystem II (PSII)[1] contains a cluster of four manganese ions. This cluster catalyzes the oxidation of two molecules of water, releasing one molecule of O_2 as a byproduct [for reviews, see Rutherford et al. (1992), Debus (1992), and Renger (1993)]. Chloride and one to two calcium ions are required for catalysis and are located near the Mn cluster. The major subunits of the PSII reaction center include an extrinsic polypeptide of 33 kDa and the membrane-spanning proteins CP47, CP43, D1, and D2 [for reviews, see Andersson and Styring (1991), Ikeuchi (1992), and Vermaas et al. (1993)]. Smaller subunits include the α and β polypeptides of cytochrome *b*-559 and the product of the *psb*I gene. The D1 and D2 polypeptides are each believed to contain five membrane-spanning α-helices and to form a heterodimer that contains the primary electron-transfer components of PSII.

Several lines of evidence suggest that the D1 polypeptide contributes many of the amino acid residues that coordinate the Mn and Ca^{2+} ions in PSII [for a review, see Debus (1992)]. On the basis of considerations of manganese coordination chemistry, the Mn ions are believed to be coordinated primarily by carboxylate residues, although serine, threonine, tyrosine, and histidine residues may participate (Pecoraro, 1988; Brudvig & Crabtree, 1989; Wieghardt, 1989). Coordination by carboxylates has been supported by chemical modification studies (Tamura et al., 1989a, 1992; Preston & Seibert, 1990, 1991a,b; Blubaugh & Cheniae, 1992) and Fourier transform infrared studies (Noguchi et al., 1992, 1993). Coordination by one or two histidine imidazole nitrogens recently has been demonstrated by ESEEM (electron spin echo envelope modulation) measurements (Tang et al., 1994a), extending earlier ESEEM (DeRose et al., 1991; Zimmermann et al., 1993) and ENDOR (electron nuclear double resonance) studies (Tang et al., 1993) that strongly implicated nitrogen ligation. Coordination by histidine had been proposed earlier on the basis of chemical modification studies (Tamura et al., 1989a; Seibert et al., 1989; Preston & Seibert, 1989, 1990, 1991a,b; Ono & Inoue, 1991) and other considerations (Kambara & Govindjee, 1985; Padhye et al., 1986). Specific amino acid residues have been proposed as ligands to Mn or Ca^{2+} on the basis of models of the structure of the lumenal regions of the D1/D2 heterodimer (Svensson et al., 1990, 1991, 1992; Ruffle & Nugent, 1992; Ruffle et al., 1992) and chemical modification and proteolysis studies (Seibert et al., 1989; Preston & Seibert, 1990, 1991b).

As an alternative approach toward the identification of amino acid residues that ligate the Mn and Ca^{2+} ions in PSII,

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[1] Abbreviations: A_{730}, optical density at 730 nm; bp, base pair; Chl, chlorophyll *a*; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Em^r, erythromycin resistant; F_{eq}, steady-state fluorescence yield produced by weak monitoring flashes in the presence of DCMU; kb, kilobase; MES, 2-morpholinoethanesulfonic acid; P_{680}, primary chlorophyll electron donor; PCR, polymerase chain reaction; PSII, photosystem II; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastoquinone electron acceptor; S_n, oxidation state of the oxygen-evolving complex with *n* oxidizing equivalents stored; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; wild-type*, control *Synechocystis* strain constructed in the same manner as site-directed mutants, but with no mutation; Y_Z, rapid electron donor to P_{680}^+ (Tyr-161 of the D1 polypeptide); Y_D, slow electron donor to P_{680}^+ (Tyr-160 of the D2 polypeptide).

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we have employed site-directed mutagenesis to individually target every conserved (Svensson et al., 1991) lumenal carboxylate, histidine, and tyrosine residue in the D1 polypeptide. We have constructed these mutations in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Nixon, Diner, and co-workers have employed a similar strategy, and both their group and ours have published preliminary accounts in recent review articles (Diner et al., 1991; Nixon et al., 1992a; Debus, 1992; Nixon & Diner, 1994). In addition, both groups (ours in collaboration with B. A. Barry and co-workers) have shown that Asp-170 is critical for the assembly or stability of the Mn cluster (Nixon & Diner, 1992; Boerner et al., 1992), serves as a component of a high-affinity binding site for a Mn^{2+} ion (Nixon & Diner, 1992; Diner & Nixon, 1992), and may serve as a ligand to the assembled Mn cluster (Nixon & Diner, 1992; Boerner et al., 1992; Diner & Nixon, 1992). Recent characterizations of Asp-170 mutants in the eukaryotic green alga *Chlamydomonas reinhardtii* are in agreement with these findings (Whitelegge et al., 1992, 1995). Nixon, Diner, and co-workers have also demonstrated that the carboxy terminus of D1 (Ala-344) is essential for the stable assembly of the Mn cluster and may serve as a ligand to the assembled cluster (Nixon et al., 1992b). Preliminary evidence presented by Nixon and Diner and by ourselves similarly implicates His-332 and Asp-342 (Diner et al., 1991; Nixon et al., 1992b; Chu et al., 1993, 1994c; Nixon & Diner, 1994).

The increased lability of the oxygen-evolving complex in several site-directed PSII mutants led us to further develop noninvasive methods for characterizing PSII mutants *in vivo* (Chu et al., 1994a). These methods involve measuring changes in the yield of chlorophyll *a* fluorescence after a single saturating flash or brief illumination given in the presence of the electron-transfer inhibitor DCMU or after each in a series of saturating flashes given in the absence of DCMU. These methods are readily applied to mutants that evolve little or no oxygen, avoid possible isolation-induced artifacts that may obscure the true nature of a mutation, and facilitate the characterization of mutants with labile oxygen-evolving complexes. On the basis of these methods, we recently determined that, in many site-directed PSII mutants, a significant fraction of PSII reaction centers lack photooxidizable[2] Mn ions *in vivo* (Chu et al., 1994a). In these reaction centers, either the high-affinity site from which Mn ions rapidly reduce Y_Z^{ox} is devoid of Mn ions or the Mn ion(s) bound at this site is(are) unable to reduce Y_Z^{ox}. In such mutants, the Mn cluster must be either unstable or assembled inefficiently *in vivo*. We also determined that the fraction of reaction centers that lack photooxidizable Mn ions *decreases* in the *absence* of the extrinsic 33 kDa polypeptide (Chu et al., 1994b), suggesting that this polypeptide may present a diffusion barrier to Mn^{2+} ions during the assembly of the Mn cluster.

In the present paper, we present the results of our *in vivo* characterizations of site-directed mutants that have substitutions in the two lumenal interhelical domains of the D1 polypeptide. These domains include the residues extending approximately from Pro-57 to Gly-109 (connecting helices A and B) and approximately from Gly-166 to His-195 (connecting helices C and D). In the following paper in this issue (Chu et al., 1995), we present the results of our *in vivo* characterizations of mutants that have substitutions in the D1 polypeptide's carboxy-terminal region. The *in vivo* characterizations of the site-directed PSII mutants described here and in the accompanying paper will guide future studies of isolated mutant PSII particles to residues that are of particular interest in terms of Mn or Ca^{2+} ligation or other aspects of PSII function and to mutants that contain the most stable or most efficiently assembled oxygen-evolving complexes.

MATERIALS AND METHODS

Growth and Preparation of Synechocystis sp. PCC 6803 Cells. *Synechocystis* cells were propagated as described previously (Chu et al., 1994a) in BG-11 growth medium (Rippka et al., 1979) supplemented with 5 mM TES−NaOH (pH 8.0) (Williams, 1988). For some experiments, the $CaCl_2$ in this medium was replaced by an equivalent concentration of $MgCl_2$ or $SrCl_2$ or by twice the concentration of NaCl (to maintain the same concentration of Cl^- ions). Such media were handled in acid-washed glassware to minimize the presence of adventitious Ca^{2+} ions. Except when the photoautotrophic growth characteristics of mutants were being investigated, the liquid media contained 5 mM glucose. Constant illumination was provided by fluorescent cool white bulbs, normally at an intensity of 50−60 μE m^{-2} s^{-1} but also, when indicated, at 5−6 μE m^{-2} s^{-1} [measured with a LI-COR (Lincoln, NE) Model LI-189 light meter]. Cells were harvested and prepared for analysis as described previously (Chu et al., 1994a).

Construction and Verification of Site-Directed Mutants. All mutants were constructed in the *psb*A-2 gene of *Synechocystis* sp. PCC 6803, as described previously (Chu et al., 1994a). The wild-type* strain, described previously (Chu et al., 1994a), was obtained by identical procedures, except that the transforming plasmid carried no mutation. The identity of each mutant was verified from the complete sequence of its *psb*A-2 gene. This sequence was obtained by directly sequencing a double-stranded 1380 bp PCR-amplified fragment of genomic DNA (Chu et al., 1994a). Homologous recombination was confirmed by analysis of Southern blots (Chu et al., 1994a). The absence of undesired mutations outside the *psb*A-2 coding region was confirmed by transforming mutants with the 594 bp *Bst*EII/*Bst*EII fragment of wild-type *psb*A-2 (Chu et al., 1994a). The innocence of silent mutations introduced to create sites for restriction endonucleases was confirmed as described previously (Chu et al., 1994a).

Where indicated, the *psb*O gene was deleted from the *Synechocystis* chromosome by replacing it with DNA encoding resistance to erythromycin (Em). The plasmid pRB-1, which contains the entire *psb*O gene of *Synechocystis* sp. PCC 6803 (Burnap & Sherman, 1991), was the generous gift of R. L. Burnap (Oklahoma State University). To replace the *psb*O gene with DNA encoding resistance to Em, the 1.36 kb *Afl*II/*Xba*I fragment of pRB-1 was replaced with the 1.53 kb *Xba*I/*Eco*RV fragment of the plasmid pRL425 (Elhai & Wolk, 1988). The resulting plasmid was transformed into *Synechocystis* cells. Transformants were selected on solid medium containing Em (0.1 μg/mL). Southern blot analysis

[2] We define "photooxidizable Mn ions" as Mn ions that are bound to PSII when actinic illumination begins and that are oxidized by Y_Z^{ox} with a half-time much shorter than that of charge recombination between Q_A^- and Y_Z^{ox}.

of genomic DNA isolated from homozygous Em^r transformants showed that the approximately 1.38 kb *StuI/XbaI* fragment bearing *psb*O in wild-type cells had increased in size to approximately 1.55 kb (data not shown).

Measurement of Photosynthetic Oxygen Evolution. Light-saturated rates of oxygen evolution were measured at 25.0 °C as described previously (Chu et al., 1994a). Samples contained 20 μg/mL Chl, 2 mM potassium ferricyanide, and 2 mM 2,6-dichloro-*p*-benzoquinone in growth medium. The zero level of oxygen evolution was established with the *Synechocystis* strain that lacks all three *psb*A genes (Debus et al., 1990). This strain exhibits an *apparent* oxygen evolution rate of 16 ± 7 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} under the conditions of our assays (Chu et al., 1994a). The oxygen evolution rates of all other strains were corrected by subtracting this presumably artifactual background.

Measurement of Chlorophyll a Fluorescence. Chlorophyll *a* fluorescence was detected with a modified Walz (Effeltrich, Germany) pulse−amplitude−modulation fluorometer [described by Schreiber (1986)], as described previously (Chu et al., 1994a). Details of the measurements are given in the figure legends and elsewhere (Chu et al., 1994a). In the presence of DCMU, the fluorescence yield elicited by the monitoring flashes rose from an initial level, F_0, to a steady-state level that we denote F_{eq}. The ratio $(F_{eq} - F_0)/(F_{max} - F_0)$ ranged from approximately 0.25 in wild-type* cells to approximately 0.05 in mutants without photooxidizable Mn ions (Chu et al., 1994a). Short dark-adaptation times were employed because some PSII mutants lose oxygen evolution activity rapidly in darkness (Chu et al., 1994a). The kinetics of the decay of fluorescence yield after a flash or continuous illumination in the presence of DCMU were analyzed using Jandel Scientific's (San Rafael, CA) PeakFit program, version 3.18. Unless stated otherwise, three exponentially decaying components were assumed (fewer components yielded nonrandom residuals).

Quantitation of PSII Reaction Centers. The relative PSII content of cells on a chlorophyll basis was estimated from the total yield of variable chlorophyll *a* fluorescence $(F_{max} - F_0)$ measured in the presence of DCMU and hydroxylamine (Philbrick et al., 1991; Nixon & Diner, 1992; Nixon et al., 1992b; Chu et al., 1994a). Measurements were performed as described previously (Chu et al., 1994a). In a previous study of 21 mutants, including eight described in this paper, the relative PSII contents estimated in this manner correlated well with those estimated from [^{14}C]DCMU binding studies (Chu et al., 1994a). Nevertheless, we will refer to the fluorescence-based estimates as "apparent" PSII contents.

RESULTS AND DISCUSSION

The lumenal interhelical domains of the D1 polypeptide contain eight conserved carboxylate residues, three conserved tyrosine residues, and two conserved histidine residues (Svensson et al., 1991). Each was individually replaced with at least one residue having different chemical/ligation properties and, if possible, with one having similar properties. To minimize the possibility of long-range structural changes, the original residues were replaced with residues of similar size.

The following mutations caused no discernible effect on either photoautotrophic growth or oxygen evolution: Y73F, Y94F, E98N, E98D, D103N, D103E, E104Q, E104D, and Y107F. We conclude that neither Tyr-73, Tyr-94, Glu-98, Asp-103, Glu-104, nor Tyr-107 is crucial to photosynthetic water oxidation under the conditions of our experiments. The reasons for the absolute conservation of these residues in all known sequences of the D1 polypeptide from cyanobacteria, algae, and higher plants (Svensson et al., 1991) remain unknown. No further characterizations of these mutants were undertaken. In contrast, most mutations constructed at Asp-59, Asp-61, Glu-65, His-92, Asp-170, Glu-189, and His-190 were deleterious to both photoautotrophic growth and oxygen evolution and were investigated further.

The remainder of the Results and Discussion section is divided into five parts. Part I describes mutations constructed at Asp-59 and Asp-61, part II describes mutations constructed at Glu-65 and His-92, and parts III, IV, and V describe mutations constructed at Asp-170, Glu-189, and His-190, respectively. In each part, the growth and fluorescence characteristics of the individual mutants are presented first, followed by an evaluation of the possible involvement of the wild-type residue in Mn or Ca^{2+} ligation or in other aspects of PSII function.

(I) Asp-59 and Asp-61

(A) Growth, Oxygen Evolution Characteristics, and PSII Contents of Mutants. The following mutants were constructed: D59E, D59N, D61E, D61N, and D61A. All five mutants were photoautotrophic (Table 1). However, D61N and D61A cells grew slowly in the absence of glucose, with A_{730} doubling times of 30−40 h compared to 13−18 h for wild-type* cells (data not shown). Neither D59N nor D61A cells grew photoautotrophically when Ca^{2+} was omitted from the BG-11 growth medium: neither Sr^{2+}, Mg^{2+}, nor Na^+ would substitute. All five mutants exhibited impaired light-saturated oxygen evolution rates (Table 1). Furthermore, the D59N, D61N, and D61A mutants lost O_2-evolving activity rapidly when illuminated with saturating light. The apparent PSII contents of all three Asp-61 mutants were similar to that of wild-type* cells, while those of D59E and D59N cells were significantly less (61−65% compared to wild type*, see Table 1). Taking into account the apparent PSII contents and considering only the initial rates of oxygen evolution, the data of Table 1 show that the PSII reaction centers in D59E, D59N, D61E, D61N, and D61A cells evolved oxygen at approximately 100%, 50%, 75%, 18%, and 20% of the rate of wild-type* reaction centers. The lower rates imply either that the catalytic efficiency of the Mn cluster is impaired or that in the D59N, D61E, D61N, and D61A mutants a significant fraction of PSII reaction centers lack photooxidizable Mn ions *in vivo*. To distinguish between these possibilities, the behavior of all five Asp-59 and Asp-61 mutants was investigated with the fluorescence assays described previously (Chu et al., 1994a).

(B) Fluorescence Characteristics of Mutants. The fluorescence yield of cyanobacteria and chloroplasts arises primarily from PSII and is governed by the redox states of Q_A (Duysens & Sweers, 1963) and P_{680} (Butler, 1972; Butler et al., 1973) [for reviews, see Krause and Weis (1991), Holzwarth (1991), and Dau (1994)]. When Q_A is fully oxidized, the fluorescence yield (F_0) is low. When Q_A is fully reduced, the fluorescence yield (F_{max}) is 2−5-fold greater than F_0. This increase comes about because the rate

Table 1: Comparison of Wild-Type* (wt*) and Mutant Strains

strain	photoautotrophic growth[a]	O_2 evolution[b] (% of wt*)	apparent PSII content[c] (% of wt*)	kinetics of Q_A^- oxidation after a single flash[d] (%)	k^{-1} (s)	no. of cultures included in analysis
wild type*	+	100	100	16 ± 4	0.10 ± 0.04	8
				43 ± 4	0.60 ± 0.13	
				41 ± 7	2.8 ± 0.4	
D59E	+	62 ± 11	61 ± 9	20 ± 3	0.08 ± 0.02	5
				42 ± 7	0.45 ± 0.10	
				38 ± 8	2.5 ± 0.5	
D59N	+	33 ± 8	65 ± 16	29 ± 9	0.15 ± 0.01	6
				53 ± 3	0.70 ± 0.16	
				18 ± 10	3.1 ± 1.4	
D61E	+	61 ± 5	80 ± 9	27 ± 7	0.09 ± 0.02	5
				38 ± 5	0.56 ± 0.07	
				34 ± 4	2.2 ± 0.2	
D61N	+ (slow)	17 ± 2	92 ± 16	23 ± 3	0.07 ± 0.02	5
				61 ± 6	0.43 ± 0.07	
				15 ± 4	1.6 ± 0.2	
D61A	+ (slow)	19 ± 6	97 ± 12	20 ± 5	0.05 ± 0.02	5
				45 ± 4	0.55 ± 0.08	
				34 ± 6	2.2 ± 0.3	
E65D	+	93 ± 11	86 ± 14	14 ± 4	0.08 ± 0.04	5
				45 ± 2	0.62 ± 0.11	
				41 ± 5	2.6 ± 0.2	
E65Q	+ (slow)	17 ± 3	94 ± 16	14 ± 2	0.08 ± 0.04	4
				45 ± 10	0.63 ± 0.06	
				41 ± 11	2.2 ± 0.2	
E65A	+ (slow)	21 ± 3	97 ± 9	14 ± 2	0.06 ± 0.02	5
				53 ± 4	0.56 ± 0.05	
				34 ± 5	2.0 ± 0.2	
H92L	+	71 ± 6	69 ± 9	32 ± 12	0.09 ± 0.01	4
				42 ± 8	0.42 ± 0.08	
				26 ± 11	3.5 ± 0.2	
D170V	+ (slow)	41 ± 10	77 ± 4	51 ± 6	0.04 ± 0.01	4
				28 ± 6	0.43 ± 0.16	
				21 ± 2	3.5 ± 0.4	
D170L	−	19 ± 4	93 ± 8	42 ± 5	0.03 ± 0.01	4
				31 ± 4	0.34 ±0.09	
				26 ± 2	3.1 ± 0.3	
D170I	−	24 ± 4	99 ± 1	42 ± 5	0.04 ± 0.01	4
				29 ± 5	0.41 ± 0.08	
				30 ± 2	2.8 ± 0.3	
E189Q	+	67 ± 5	95 ± 12	20 ± 4	0.08 ± 0.02	6
				37 ± 4	0.67 ± 0.09	
				43 ± 5	3.1 ± 0.4	
E189D	−	0	108 ± 6	17 ± 3	0.07 ± 0.02	6
				44 ± 5	0.64 ± 0.11	
				39 ± 6	2.8 ± 0.3	
E189N	−	6 ± 2	121 ± 6	18 ± 1	0.06 ± 0.01	4
				57 ± 3	0.60 ± 0.04	
				25 ± 4	3.4 ± 0.4	
H190R	−	13 ± 2	124 ± 14	39 ± 7	0.08 ± 0.02	4
				31 ± 8	1.9 ± 0.3	
				31 ± 6	12 ± 2	
H190N	−	0	107 ± 11	43 ± 10	0.18 ± 0.06	5
				57 ± 10	16 ± 2	
H190Q	−	0	110 ± 20	57 ± 3	0.11 ± 0.02	5
				43 ± 3	14 ± 3	
H190L	−	0	55 ± 9	56 ± 2	0.24 ± 0.06	3
				44 ± 2	15 ± 2	
H190Y	−	0	61 ± 4	56 ± 6	0.16 ± 0.09	3
				44 ± 6	10 ± 1	

[a] Measured in growth medium without glucose. [b] Initial rates measured in growth medium. The eight wild-type* cultures used in this study evolved 680 ± 30 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (Chu et al., 1994a). [c] Estimated from the total yield of variable chlorophyll *a* fluorescence ($F_{max} - F_0$). For D61E, D61N, D61A, E65D, E65Q, E65A, E189D, and E189Q cells, these estimates have been shown to correlate with those estimated from [^{14}C]DCMU binding studies (Chu et al., 1994a). For the other mutants listed, this correlation is assumed. [d] Measured in the presence of DCMU and analyzed by assuming three exponentially decaying components (except for the H190N, H190Q, H190L, and H190Y mutants, where two components were assumed). The relative amplitude (%) and the inverse of the rate constant of each component are reported. See the left panels of Figures 2, 6, 8, 11, and 13.

constant of charge separation decreases when Q_A is reduced (Schatz et al., 1988; Leibl et al., 1989; Roelofs et al., 1992), presumably because of the electrostatic influence of Q_A^- on Pheo$^-$ (Schatz et al., 1988). The diminished rate constant decreases the yield of charge separation, thereby increasing the lifetime of the excited state of the antenna chlorophylls,

from which the fluorescence emanates. The state $Y_ZP_{680}^+Q_A^-$ is formed 300−500 ps after the excitation of P_{680} [for reviews, see Renger (1992) and Diner and Babcock (1995)]. In spite of the presence of Q_A^-, this state exhibits a low yield of chlorophyll *a* fluorescence because P_{680}^+ quenches the fluorescence. The subsequent reduction of P_{680}^+ by Y_Z [in 20−280 ns if the native Mn cluster is present (Brettel et al., 1984; Meyer et al., 1989) or in 20−40 μs if the Mn cluster is absent (van Best & Mathis, 1978; Brettel et al., 1984)] generates the highly fluorescent state $Y_Z^{ox}P_{680}Q_A^-$.

(i) Electron Transfer to Y_Z^{ox}. The different fluorescence yields of the two states $Y_ZP_{680}^+Q_A^-$ and $Y_Z^{ox}P_{680}Q_A^-$ form the basis of an assay that is sensitive to either the absence of photooxidizable Mn ions in PSII or slow electron donation from Mn to Y_Z^{ox} (Nixon & Diner, 1990, 1992; Boerner et al., 1992; Chu et al., 1994a). This assay involves the measurement of the maximum yield of chlorophyll *a* fluorescence that follows each flash in a closely spaced series of saturating flashes given in the absence of DCMU. The *first* saturating flash generates the highly fluorescent state $Y_Z^{ox}P_{680}Q_A^-$ (see preceding paragraph). Normally, the Mn cluster reduces Y_Z^{ox} within 30−1300 μs (Dekker et al., 1984). The subsequent oxidation of Q_A^- by Q_B generates the state $Y_ZP_{680}Q_AQ_B^-$, returning the fluorescence yield to F_0 (Bowes & Crofts, 1980). The *second* saturating flash then regenerates the highly fluorescent state $Y_Z^{ox}P_{680}Q_A^-$. However, if the Mn cluster is absent, or if electron transfer from Mn to Y_Z^{ox} is slowed considerably, the state $Y_Z^{ox}P_{680}Q_AQ_B^-$ will remain present when the *second* saturating flash is applied. This flash then forms the low-fluorescent state $Y_Z^{ox}P_{680}^+Q_A^-Q_B$. Because P_{680}^+ cannot be reduced by Y_Z^{ox}, the maximum fluorescence yield after the *second* saturating flash will be quenched.

The fluorescence yields that followed each of four saturating flashes spaced 50 ms apart are shown in Figure 1 for the mutants D59N, D61N, and D61A. In D59N cells (Figure 1A), the maximum fluorescence yield after each saturating flash was essentially the same given the signal-to-noise ratio of our data. Similar data were obtained for wild-type*, D59E, and D61E cells (not shown). In contrast, in D61N and D61A cells (Figure 1B,C, respectively), the maximum fluorescence yields after the second and subsequent flashes were partly quenched. The extent of quenching diminished as the spacing between the saturating flashes was increased, becoming negligible at flash spacings of 200−400 ms (not shown). These data show that the reduction of Y_Z^{ox} is slowed dramatically in the D61N and D61A mutants: a considerable fraction of Y_Z^{ox} remains oxidized for at least 50 ms after each saturating flash (Chu et al., 1994a). These results imply that either significant fractions of reaction centers in these mutants lack photooxidizable Mn ions or electron transfer from Mn to Y_Z^{ox} is slowed considerably during one or more of the S-state transitions.

In D61A cells, some Q_A^- remained reduced 50 ms after each flash (Figure 1C). The amount decreased as the spacing between flashes increased (not shown), showing that the slowest components of electron transfer from Q_A^- to Q_B were slowed in this mutant. Mutations of Asp-170 also slow this electron-transfer step (Chu et al., 1994a). Other workers have noted that many treatments that alter the donor side of PSII also alter the properties of Q_A and/or Q_B [*e.g.*, see Krieger and Weis (1992), Krieger et al. (1993), Johnson et al. (1995), and references cited in Chu et al. (1994a)].

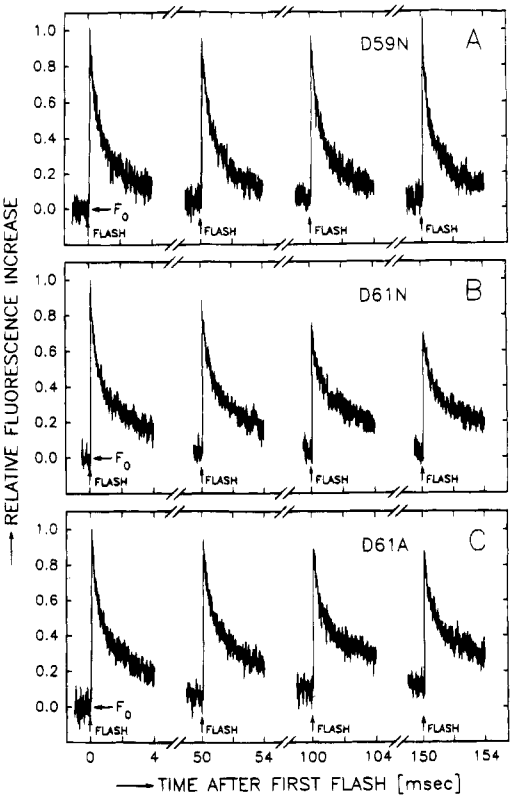

FIGURE 1: Yields of variable chlorophyll *a* fluorescence produced by each of four saturating flashes given at 50 ms intervals to Asp-59 and Asp-61 mutant cells. Five milliseconds of data are shown for each flash. (A) D59N, (B) D61N, (C) D61A. Conditions: 20 μg of Chl in 0.58 mL of 50 mM MES−NaOH, 25 mM $CaCl_2$, and 10 mM NaCl, pH 6.5, 22 °C. The cells were incubated in darkness for 1 min before the monitoring flashes were switched on. The frequency of the monitoring flashes was switched from 1.6 to 100 kHz for 5 ms beginning 1 ms before each saturating flash. The vertical scales are normalized to the maximum $(F - F_0)/F_0$ values measured after the first flash in each series. These values were 0.31 for (A), 0.46 for (B), and 0.45 for (C). The data of D59E and D61E cells (not shown) resembled those of D59N cells.

(ii) Charge Recombination between Q_A^- and PSII Electron Donors. An alternate assay that is sensitive to the absence of photooxidizable Mn ions in PSII is based on measuring the kinetics of charge recombination between Q_A^- and PSII electron donors after a single saturating flash (Nixon & Diner, 1990, 1992; Boerner et al., 1992; Chu et al., 1994a). In *Synechocystis* 6803, the yield of variable chlorophyll *a* fluorescence is believed to be proportional to the concentration of Q_A^- (Philbrick et al., 1991; Nixon et al., 1992b). Consequently, the kinetics of charge recombination can be measured from the decay of fluorescence yield that follows a saturating flash given in the presence of DCMU. Data for wild-type*, D59N, D61N, and D61A cells are shown in Figure 2A−D, respectively. The data for D59E and D61E cells (not shown) resembled those of D61A cells (Figure 2D), except that the maximal $[(F - F_{eq})/(F_{max} - F_{eq})]$ ratios were higher (the ratios were approximately 0.90 for both D59E and D61E cells). The amplitudes and rates of the decay components are presented in Table 1. None of the Asp-59 or Asp-61 mutants exhibited significant amounts of rapid decay kinetics that could correspond to charge recombination between Q_A^- and Y_Z^{ox}. These results suggest that essentially all PSII reaction centers in these mutants contain photooxidizable Mn clusters. However, in all five mutants, particularly in D59N (Figure 2B) and D61N (Figure 2C) cells, the

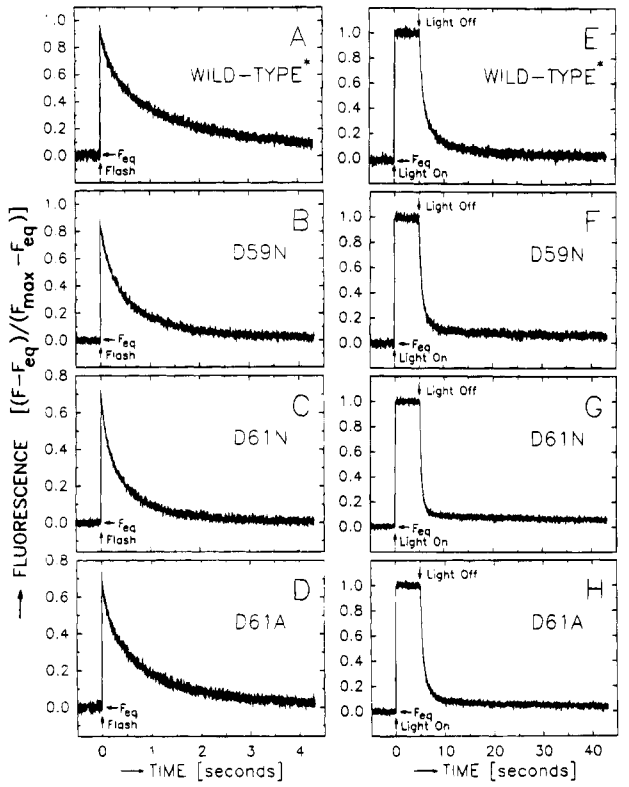

FIGURE 2: Formation and decay of Q_A^- in response to a saturating flash (left panels) or to 5 s of illumination (right panels) given to wild-type*, Asp-59, and Asp-61 mutant cells in the presence of DCMU, as measured by changes in the yield of chlorophyll *a* fluorescence: (A, E) wild-type*; (B, F) D59N; (C, G) D61N; (D, H) D61A. Note the 10-fold difference in time scale between the left and right panels. The conditions were the same as in Figure 1 except that the samples were incubated in darkness for 1 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 concentration of ethanol was 2%). For a definition of F_{eq}, see Materials and Methods. The data in each left panel were normalized with the F_{max} value determined in the corresponding right panel. The F_{max} values (right panels) were not increased by the addition of hydroxylamine. Each trace in (A–D) represents the computer average of 8–10 traces. The monitoring flashes were applied at 1.6 kHz. The data of D59E and D61E cells (not shown) resembled those of D61A cells.

decay of fluorescence yield was more rapid than that in wild-type* cells. The observed rate of charge recombination is determined by the rate of charge recombination between Q_A^- and P_{680}^+ [$\tau_{1/2} \approx 900$ μs in *Synechocystis* 6803 (Metz et al., 1989)] and the equilibrium concentration of P_{680}^+. In PSII reaction centers having an intact Mn cluster, the concentration of P_{680}^+ is determined by the equilibrium between S_2P_{680} and $S_1P_{680}^+$ (Bouges-Bocquet, 1980; Buser et al., 1992). By assuming that charge recombination between Q_A^- and P_{680}^+ is not altered in the mutants, the more rapid charge recombination kinetics indicates that the equilibrium concentration of P_{680}^+ is higher in the mutants than in wild-type* cells. This implies that the S_2/S_1 midpoint potential of the Mn cluster increased slightly in all five mutants, particularly in D59N and D61N.

(iii) Photoaccumulation of Q_A^-. We recently presented evidence that the fraction of reaction centers without photooxidizable Mn ions in site-directed PSII mutants can be estimated from the rate at which Q_A^- photoaccumulates when cells are briefly illuminated in the presence of DCMU (Chu et al., 1994a). The basis for this assay is that cytochrome

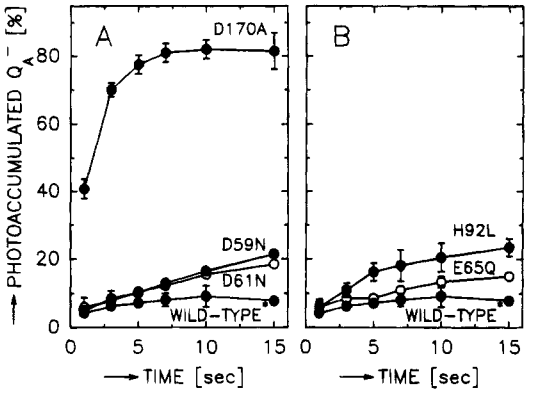

FIGURE 3: Fraction of PSII reaction centers in Asp-59, Asp-61, Glu-65, and His-92 mutant cells that photoaccumulated Q_A^- after illumination for specific intervals of time. This fraction is defined as the fraction of reaction centers in which Q_A^- was oxidized with a time constant of 1–2 min (*e.g.*, see Figure 2E–H and text). (A) Photoaccumulation of Q_A^- in D59N and D61N cells in comparison to wild-type* and D170A cells. (B) Photoaccumulation of Q_A^- in E65Q and H92L cells in comparison to wild-type* cells. The data of D59E, D61E, and D61A cells (not shown) resembled those of D59N and D61N cells. The data of E65D and E65A cells (not shown) resembled those of E65Q cells. The wild-type* and D170A data are taken from Chu et al. (1994a) to facilitate comparisons. The conditions were the same as in Figure 2.

b-559 and Y_D are oxidized by P_{680}^+ with low quantum yields (Buser et al., 1990, 1992; Vass & Styring, 1991; Buser, 1993). The rate of oxidation correlates with the equilibrium concentration of P_{680}^+. Consequently, both cytochrome *b*-559 and Y_D are oxidized far more rapidly in PSII reaction centers that *lack* photooxidizable Mn ions than in reaction centers that *contain* photooxidizable Mn ions. Both oxidized cytochrome *b*-559 and Y_D^{ox} are relatively stable species, even in the presence of Q_A^-. During continuous illumination in the presence of DCMU, the states $Y_Z^{ox}Q_A^-$ and $S_2Q_A^-$ form and recombine until the stable states $cyt^{ox}Q_A^-$ and $Y_D^{ox}Q_A^-$ photoaccumulate. The subsequent oxidation of Q_A^- is a slow process (τ > 1 min).

To estimate the fractions of reaction centers that lack photooxidizable Mn ions in the Asp-59 and Asp-61 mutants *in vivo*, cells were illuminated for 1–15 s in the presence of DCMU. The kinetics of Q_A^- oxidation in wild-type*, D59N, D61N, and D61A cells after 5 s of illumination are presented in Figure 2E–H. The data of D59E and D61E cells (not shown) resembled those of D59N cells. In all five mutants, the rapidly decaying components were slightly more rapid than those in wild-type* cells, in agreement with the slightly more rapid $S_2 \rightarrow Q_A^-$ charge recombination kinetics observed following a single flash (Figure 2A–D). Importantly, the fraction of Q_A^- that photoaccumulated in each mutant during 5 s of illumination (*i.e.*, the fraction that oxidized slowly, with a time constant of 1–2 min) was only 12–13% of the total, similar to the fraction observed in wild-type* cells [7.2% ± 0.9% (Chu et al., 1994a)]. Figure 3A shows the fractions of Q_A^- that photoaccumulated during 1–15 s of illumination in D59N and D61N cells in comparison to wild-type* cells. Similar data were exhibited by the D59E, D61E, and D61A mutants (not shown). Data for the D170A mutant, which lacks photooxidizable Mn ions (Chu et al., 1994a), are shown for comparison. In all five mutants, the Q_A^- photoaccumulation rate was only slightly greater than that in wild-type* cells. The slightly more rapid photoaccumulation rates are consistent with the slightly

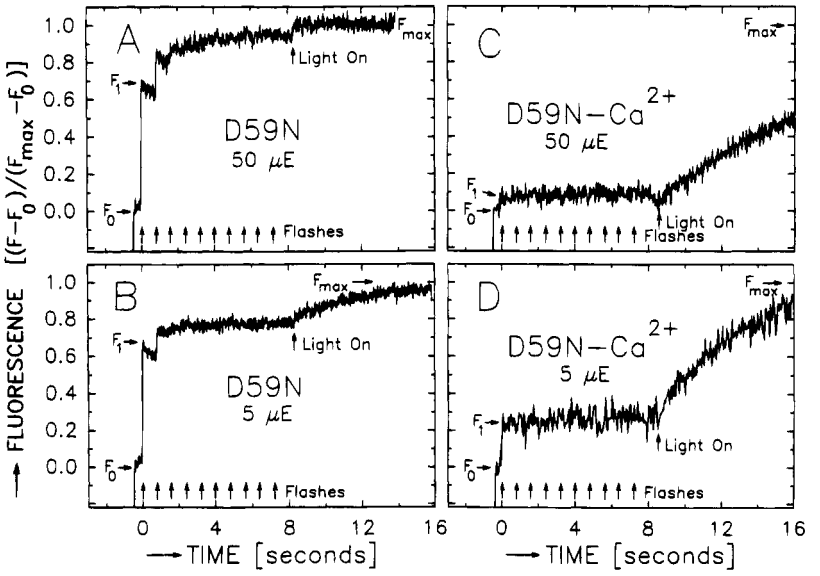

FIGURE 4: Formation of Q_A^- in D59N cells in response to 10 saturating flashes given at 800 ms intervals in the presence of DCMU and hydroxylamine, followed by continuous illumination: (A) cells propagated in the *presence* of Ca^{2+} ions at 50−60 μE m^{-2} s^{-1}; (B) cells propagated in the *presence* of Ca^{2+} ions at 5−6 μE m^{-2} s^{-1}; (C) cells propagated in the *absence* of Ca^{2+} ions at 50−60 μE m^{-2} s^{-1}; (D) cells propagated in the *absence* of Ca^{2+} ions at 5−6 μE m^{-2} s^{-1}. The conditions were the same as in Figure 2 except that the cells were incubated in darkness in the presence of *p*-benzoquinone and potassium ferricyanide for 5 min before the addition of DCMU. Hydroxylamine (20 mM) was added 1 min after the DCMU. The monitoring flashes were switched on 2 min after the addition of hydroxylamine, followed 0.5 s later by the saturating flashes (arrows). Continuous illumination (applied to obtain F_{max}) was applied 1.1 s after the tenth flash (arrow). The initial fluorescence yields produced by the monitoring flashes (F_0) are indicated. The monitoring flashes were applied at 1.6 kHz. The data presented in (A) are essentially the same as those for wild-type* cells (not shown).

higher equilibrium concentrations of P_{680}^+ inferred to be present in these mutants (see above). Importantly, little or no Q_A^- photoaccumulated within the first second of illumination. These data confirm that essentially all PSII reaction centers in the Asp-59 and Asp-61 mutants contain intact, photooxidizable Mn clusters whose S_2/S_1 redox properties differ only slightly from those of wild-type Mn clusters.

(iv) Flash-Induced Increases in Fluorescence Yield. In all of the Asp-59 and Asp-61 mutants, the increase in fluorescence yield produced by a saturating flash (relative to F_{max}) was slightly lower than that in wild-type* cells (Figure 2). One possible explanation for the lower flash-induced increases is that electron transfer from Y_Z to P_{680}^+ is slowed in the mutants, resulting in a lower quantum yield for the formation of the highly fluorescent state $Y_Z^{ox}P_{680}Q_A^-$. To test this possibility, we measured the increase in yield produced by the first in a series of saturating flashes given in the presence of DCMU and hydroxylamine (Metz et al., 1989; Chu et al., 1994a). Hydroxylamine reduces Y_Z^{ox} (Bennoun, 1970), trapping Q_A^-, and at the concentrations we employ (20 mM), it extracts the Mn cluster (Cheniae & Martin, 1971b). The rate of electron transfer from Y_Z to P_{680}^+ should, therefore, be 20−40 μs in *all* cells (see above) unless the rate is altered by a mutation. Data for the D59N mutant are shown in Figure 4A [the data are normalized to ($F_{max} - F_0$) rather than ($F_{max} - F_{eq}$) because stable F_{eq} levels cannot be achieved in our apparatus in the presence of hydroxylamine (Chu et al., 1994a)]. The ratio ($F_1 - F_0$)/($F_{max} - F_0$) in all of the Asp-59 and Asp-61 mutants was similar to that in wild-type* cells. [The ratios were 0.65 ± 0.06 for D59E cells (not shown), 0.69 ± 0.04 for D59N cells (Figure 4A), 0.56 ± 0.05 for D61E cells (not shown), 0.66 ± 0.06 for D61N cells (not shown), and 0.64 ± 0.06 for D61A cells (not shown), compared to 0.59 ± 0.06 for wild-type* cells (Chu et al., 1994a).] Consequently, the lower flash-induced increases in fluorescence yield observed in the mutants in the *absence* of hydroxylamine (Figure 2B−D) were not caused by slowed electron transfer from Y_Z to P_{680}^+, unless this electron-transfer step is slowed substantially (relative to wild type) only in the *absence* of hydroxylamine.

The lower flash-induced fluorescence yields in the absence of hydroxylamine correlated with accelerated $Q_A^- \rightarrow S_2$ charge recombination kinetics in the D59E, D59N, D61E, and D61N mutants (*e.g.*, see Figure 2B,C). Consequently, the lower flash-induced yields in these mutants may be caused by the higher equilibrium concentrations of P_{680}^+ inferred to be present. The lower flash-induced yield in D61A cells (Figure 2D) does not fit this correlation, however. The same or higher flash-induced yields are observed in D59N and D61N (Figure 2B,C), two mutants that exhibit more rapid charge recombination kinetics than D61A. Consequently, the lower flash-induced yield observed in D61A cells may be caused, in part, by the presence of a fluorescence quencher other than P_{680}^+. The presence of such a quencher has been inferred previously in the mutants Δ*psb*O and D170R (Philbrick et al., 1991; Chu et al., 1994a).

(v) Requirement for Ca^{2+} Ions. We noted earlier that neither D59N nor D61A cells grew photoautotrophically in media containing Sr^{2+}, Mg^{2+}, or Na^+ substituted for Ca^{2+}. Cells propagated photoheterotrophically (in the presence of 5 mM glucose) in such media exhibited little or no oxygen evolution (0−7%, depending on culture) and only small increases in fluorescence yield when subjected to a saturating flash in the presence of DCMU (not shown). The flash-induced increases were larger in cells propagated in dim light (5−6 μE m^{-2} s^{-1}) than in cells propagated in normal light (50−60 μE m^{-2} s^{-1}), suggesting that Ca^{2+}-poor D59N and D61A cells are extremely light-sensitive. The charge recombination kinetics observed in these cells after a single

saturating flash was slow (not shown), superficially resembling that observed in cells that lack the extrinsic 33 kDa polypeptide (Chu et al., 1994b). These results suggest that the Ca^{2+}-poor PSII reaction centers in D59N and D61A cells propagated in the absence of Ca^{2+} ions contain altered or partly assembled Mn clusters. The small increases in fluorescence yield produced by saturating flashes precluded a more detailed investigation of this point, however. To determine whether the small flash-induced increases in fluorescence yield might be caused by slowed electron transfer from Y_Z to P_{680}^+, the increases produced by flashes given in the presence of DCMU and hydroxylamine were measured. For these experiments, cells were propagated photoheterotrophically in both normal and dim light. In D59N cells propagated in normal light with Ca^{2+} replaced by Na^+, the $(F_1 - F_0)/(F_{max} - F_0)$ ratio was approximately 0.08 (Figure 4C). When the cells were propagated in dim light, the ratio was approximately 0.25 (Figure 4D). Similar data were acquired with the D61A mutant (not shown). These ratios suggest that electron transfer from Y_Z to P_{680}^+ is slowed dramatically in Ca^{2+}-poor D59N and D61A cells and that these cells are extremely light-sensitive. Indeed, the increases in fluorescence yield produced by the *second* and subsequent flashes (and by continuous illumination) were substantially *less* in Ca^{2+}-poor D59N and D61A cells (*e.g.*, see Figure 4C,D) than in a mutant that lacks Y_Z [*e.g.*, in the mutant Y161F (Debus et al., 1988; Metz et al., 1989)], even when the Ca^{2+}-poor D59N and D61A cells were propagated in dim light (see the section on His-190 mutants to follow). Consequently, some light-induced damage must have occurred in these cells even at light intensities of 5−6 μE m^{-2} s^{-1}. In contrast, D59N and D61A cells propagated in the *presence* of Ca^{2+} ions exhibited no apparent light sensitivity (*e.g.*, compare Figure 4A,B), except when illuminated with the saturating light used in oxygen evolution assays (see above). Surprisingly, however, the apparent PSII contents of Ca^{2+}-poor D59N and D61A cells were diminished when the cells were propagated in dim light: from approximately 35% to 17% in D59N cells and from approximately 100% to 45% in D61A cells (not shown).

(C) Involvement in Mn Binding. From the results presented earlier, we conclude that neither Asp-59 nor Asp-61 is essential for the assembly or stability of the Mn cluster. Therefore, it is unlikely that either residue ligates Mn. The slow kinetics of charge recombination and rates of Q_A^- photoaccumulation in the mutants D59N, D61N, and D61A (Figures 2 and 3A) show that these mutants contain few, if any, PSII reaction centers that lack photooxidizable Mn ions. The progressive quenching of the maximum yields of fluorescence following the second and subsequent flashes in D61N and D61A cells (Figure 1B,C) indicates, however, that electron transfer from the Mn cluster to Y_Z^{ox} must be slowed substantially during one or more of the S-state transitions. The absence of apparent quenching in the D59N mutant implies that none of the S-state transitions is slowed dramatically in this mutant. However, the lower light-saturated rate of oxygen evolution in this mutant (Table 1) implies that the rate of at least one S-state transition or of O_2 release is slowed to some extent. Wild-type cells that lack the extrinsic 33 kDa polypeptide [*e.g.*, Δ*psb*O cells (Burnap & Sherman, 1991; Philbrick et al., 1991)] are similar to D59N cells in that both evolve oxygen at approximately 33% of the rate of wild-type cells, yet exhibit no progressive quenching of the maximal fluorescence yield (Philbrick et al., 1991; Chu et al., 1994b). When the extrinsic 33 kDa polypeptide is removed from spinach PSII membranes or deleted from wild-type *Synechocystis* cells, none of the S-state transitions is slowed dramatically (Miyao et al., 1987), but the kinetics of O_2 release is slowed by approximately 2.5-fold (Miyao et al., 1987; Burnap et al., 1992).

That partial quenching is evident in D61N and D61A cells following the second flash (Figure 1B,C) might suggest that electron transfer from Mn to Y_Z^{ox} is slowed during the $S_1 \rightarrow S_2$ transition. However, because the cells were not extensively dark-adapted, and because the weak monitoring flashes were applied at 100 kHz for 5 ms surrounding each saturating flash, it seems likely that, in many PSII reaction centers, the Mn cluster would have been in the S_2 or S_3 state prior to the firing of the first saturating flash. Indeed, because little or no $Q_A^- \rightarrow Y_Z^{ox}$ charge recombination was observed in these mutants after a single flash given in the presence of DCMU (Figure 2C,D, Table 1), we suggest that electron transfer from Mn to Y_Z^{ox} is slowed substantially during either the $S_2 \rightarrow S_3$ or $S_3 \rightarrow (S_4) \rightarrow S_0$ transition, rather than during the $S_1 \rightarrow S_2$ transition. It should be possible to test this assertion with O_2 flash yield experiments by individually changing the times between the first four flashes in a series and by measuring the kinetics of O_2 release following the third flash [*e.g.*, see Kok et al. (1970), Bouges-Bocquet (1973), and Miyao et al. (1987)].

(D) Involvement in Ca^{2+} Binding. That neither D59N nor D61A cells grew photoautotrophically when Ca^{2+} was replaced by Sr^{2+}, Mg^{2+}, or Na^+ indicates that the affinity of PSII for Ca^{2+} in these mutants is too low to utilize the quantities of adventitious Ca^{2+} present in our Ca^{2+}-depleted growth media. These quantities *are* sufficient to sustain the normal growth of wild-type* cells and most mutants, however. We conclude that both Asp-59 and Asp-61 influence the binding of Ca^{2+} to PSII. The small increases in fluorescence yield induced by saturating flashes in the presence of hydroxylamine and DCMU (Figure 4C,D) indicate that electron transfer from Y_Z to P_{680}^+ is slowed significantly in Ca^{2+}-poor D59N and D61A cells. Slowed electron transfer from Y_Z to P_{680}^+ has been observed previously in PSII preparations from a cyanobacterium (Satoh & Katoh, 1985) and from spinach (Boussac et al., 1992) after rigorous depletion of Ca^{2+} ions.

We suggest that Asp-59 and Asp-61 may directly coordinate a Ca^{2+} ion in PSII. Several workers have previously included these residues in models for Ca^{2+} binding (Dismukes, 1988; Ono & Inoue, 1989; Yocum, 1991). Indeed, both residues may compose part of an Asx turn, a motif found in Ca^{2+} binding loops in most Ca^{2+} binding proteins whose structures are known to high resolution (McPhalen et al., 1991). An Asx turn involves a hydrogen bond between a side chain oxygen of (usually) an Asp or Asn residue at position n and the peptide amide nitrogen at position $n + 2$ (Rees et al., 1983). In most Ca^{2+} binding proteins, the Asx residue is Asp. Furthermore, a Pro or Gly residue is usually located immediately adjacent to the turn and within three residues of the Asx residue (McPhalen et al., 1991). These residues act in conjunction with the Asx turn to fold the Ca^{2+} binding loop around the Ca^{2+} ion and to correctly position the oxygen ligands. Both Asp-59 and Asp-61 are located near the center of a 14-residue stretch that is conserved in

all known D1 sequences (Svensson et al., 1991) and includes Pro-56, Pro-57, and Gly-62. Therefore, Asp-59 is an excellent candidate to be an Asx residue in a Ca^{2+} binding loop. If this residue normally coordinates a Ca^{2+} ion with both side chain oxygens, then the replacement of Asp with Asn would likely decrease the affinity of the site for Ca^{2+} ions [*e.g.*, see Maune et al. (1992) and Babu et al. (1992)]. Similarly, if Asp-61 coordinates a Ca^{2+} ion via a single oxygen, replacement of this residue with Asn may not seriously weaken binding [*e.g.*, see Babu et al. (1992)], but its replacement with Ala would [*e.g.*, see Reinach et al. (1986), Putkey et al. (1989), Geiser et al. (1991), and Babu et al. (1992)].

The residues Asp-59 and Asp-61 are located near the base of the A helix. If the helices of the D1/D2 heterodimer are arranged as in the LM units of reaction centers from purple non-sulfur bacteria, then these residues must be located at least 15 Å from Asp-170. Indeed, existing models for PSII place Asp-59 and Asp-61 at even greater distances from this residue (Svensson et al., 1990, 1991, 1992; Ruffle & Nugent, 1992; Ruffle et al., 1992). The possibility that Asp-59 and Asp-61 ligate Ca^{2+} in PSII raises an apparent dilemma: if Asp-170 is located near the Mn cluster [as expected on the basis of the work of Nixon and Diner (1992) and Boerner et al. (1992)] and the PSII core contains only one Ca^{2+} ion (Han & Katoh, 1993), then ligation of Ca^{2+} by Asp-59 and Asp-61 would place Ca^{2+} far from the Mn cluster. However, it is widely presumed that the Mn cluster and Ca^{2+} are located in close proximity in PSII. This presumption is based on the altered line shapes of the S_2-state multiline EPR signal in Ca^{2+}-depleted (Boussac et al., 1989) and Sr^{2+}-repleted (Boussac & Rutherford, 1988) samples, on the stabilizing effects of Ca^{2+} on the ligation of over-reduced forms of the Mn cluster (Mei & Yocum, 1991), on a report that a Ca^{2+} site is created during assembly of the Mn cluster (Tamura & Cheniae, 1988), and on interpretations of X-ray absorption data that yield Mn−Ca^{2+} distances of approximately 3.3−3.4 (Yachandra et al., 1993; DeRose et al., 1994), 3.6−3.7 (MacLachlan et al., 1992, 1994), or possibly 4.3−4.4 Å (Penner-Hahn et al., 1990). There are a number of possible resolutions to this apparent dilemma, however.

First, the Mn cluster and Ca^{2+} may *not* be located in close proximity. Most of the phenomena mentioned in the preceding paragraph could result from long-range conformational changes associated with Ca^{2+} binding or removal. Significant structural changes take place in many Ca^{2+} binding proteins when Ca^{2+} binds (Strynadka & James, 1991). Furthermore, in the most definitive X-ray absorption study to address the question of Ca^{2+} binding, the Mn−Ca^{2+} distance was inferred from differences in scattering amplitudes between Ca^{2+}-depleted samples reconstituted with Sr^{2+} or Ca^{2+} (Yachandra et al., 1993). These differences have not been observed by other workers, however (C. F. Yocum and J. E. Penner-Hahn, personal communication), raising the possibility that Mn and Ca^{2+} may not be in close proximity. A second possible resolution is that there may be two Ca^{2+} ions in the PSII core [*e.g.*, see Kalosaka et al. (1990)]: the number of Ca^{2+} ions in PSII has long been controversial [for reviews, see Yocum (1991), Debus (1992), and Boussac and Rutherford (1994)]. Finally, a third possibility is that Asp-59 and Asp-61 may not ligate Ca^{2+} directly, but that mutations at these positions cause structural changes that weaken the affinity of Ca^{2+} for a site located some distance away. In chloramphenicol acetyltransferase, the Asp-199 → Asn mutation disrupts a buried salt bridge, resulting in the movement of two polypeptide loops, including residues 20 Å from the mutation site (Gibbs et al., 1990). However, if such structural changes account for weakened Ca^{2+} binding in the D59N and D61A mutants, they do not appear to drastically alter the electron-transfer rates between Y_Z and P_{680}^+ (Figure 4A) or between the Mn cluster and Y_Z^{ox} (at least not in D59N cells, Figure 1A) or to alter the apparent S_2/S_1 midpoint potential of the Mn cluster (Figure 2B,D). Further spectroscopic studies will be required to differentiate between these possibilities.

(E) Photoinhibition in the Absence of Ca^{2+} Ions. The extreme light sensitivity of D59N and D61A cells propagated in the absence of Ca^{2+} ions (Figure 4C,D) suggests the possibility of donor side photoinhibition [for reviews of photoinhibition in PSII, see Prášil et al. (1992) and Aro et al. (1993)]. This type of photoinhibition is believed to involve the accumulation of highly oxidizing radicals on the donor side of PSII [*e.g.*, see Eckert et al. (1991) and Blubaugh et al. (1991)]. However, we do not observe similar light sensitivity in Asp-170 mutants that lack photooxidizable Mn ions [*e.g.*, in D170A, D170N, or D170T cells (Chu et al., 1994a)]. There are two possible explanations for the disparate light sensitivities of Ca^{2+}-poor D59N and D61A cells and the Asp-170 mutants. First, the extreme light sensitivity of Ca^{2+}-poor D59N and D61A cells may be caused by structural changes associated with the absence of a Ca^{2+} ion. Structural changes have been proposed to occur in PSII in the absence of Ca^{2+} [*e.g.*, see Krieger and Weis (1992), Krieger et al. (1993), and Johnson et al. (1995)]. However, if such structural changes account for the extreme light sensitivity of Ca^{2+}-poor D59N and D61A cells, then the absence of extreme light sensitivity in the Asp-170 mutants implies that a Ca^{2+} site is occupied in these mutants in the absence of the Mn cluster. Because the binding of Ca^{2+} is believed to occur *after* the assembly of the Mn cluster (Tamura & Cheniae, 1988; Miller & Brudvig, 1989; Tamura et al., 1989b), this would imply the existence of at least two Ca^{2+} sites in the PSII core. A second explanation for the extreme light sensitivity of Ca^{2+}-poor D59N and D61A cells is that, when oxidized, the altered or partly assembled Mn clusters in these cells give off activated oxygen species such as hydrogen peroxide. We believe that such a photoinhibitory mechanism occurs in numerous site-directed mutants that have substitutions in the D1 polypeptide's carboxy-terminal region [for a detailed discussion of this point, see the following paper in this issue (Chu et al., 1995)].

(II) Glu-65 and His-92

(A) Growth, Oxygen Evolution Characteristics, and PSII Contents of Mutants. The following mutants were constructed: E65D, E65Q, E65A,[3] and H92L. All four mutants were photoautotrophic (Table 1). However, E65Q and E65A

[3] We recently discovered that the data we previously reported for E65A cells (Chu et al., 1994a) were actually obtained with D342E cells. The four cultures of E65A examined originated from a mislabeled frozen glycerol stock. The similarity between the E65A and D342E data presented in Chu et al. (1994a) is, therefore, more than coincidental. The point illustrated by these data (that the Q_A^- photoaccumulation rate appears to correlate with the equilibrium concentration of P_{680}^+ in mutants that have intact Mn clusters) remains valid, however, as illustrated by data presented in this paper.

cells grew slowly in the absence of glucose, with A_{730} doubling times of 30−40 h compared to 13−18 h for wild-type* cells (data not shown). The photoautotrophic growth rates of the Glu-65 mutants were not significantly affected when Ca^{2+} was omitted from the growth medium. In contrast, H92L cells exhibited little or no growth when Ca^{2+} was replaced with Na^+, grew slowly when Ca^{2+} was replaced with Mg^{2+} (with a doubling time of approximately 25 h), and grew at normal rates when Ca^{2+} was replaced with Sr^{2+} (data not shown). The E65Q, E65A, and H92L mutants exhibited impaired light-saturated oxygen evolution rates (Table 1). However, only E65Q and E65A cells rapidly lost O_2-evolving activity when illuminated with saturating light. The apparent PSII contents of all three Glu-65 mutants were similar to that of wild-type* cells, while that of H92L cells was significantly less (approximately 70% compared to wild type*, see Table 1). Taking into account the apparent PSII contents and considering only the initial rates of oxygen evolution, the data of Table 1 show that the PSII reaction centers in E65D and H92L cells evolved oxygen at approximately the same rates as wild-type* reaction centers, while those in E65Q and E65A cells evolved oxygen at only 18−20% of the wild-type* rate.

(B) Fluorescence Characteristics of Mutants. (i) Electron Transfer to Y_Z^{ox}. The fluorescence yields that followed each of four saturating flashes spaced 50 ms apart are shown in Figure 5 for the mutants E65Q, E65A, and H92L. Little or no quenching of the maximal yield was observed in these mutants after any flash. Similar data were obtained for the E65D mutant (not shown). These results show that electron transfer to Y_Z^{ox} is not slowed dramatically in H92L or in any of the Glu-65 mutants during any of the S-state transitions.

In both E65Q and E65A cells, some Q_A^- remained reduced 50 ms after each flash (Figure 5A,B). The amount decreased as the spacing between flashes increased (not shown), showing that the slowest components of electron transfer from Q_A^- to Q_B were slowed in these mutants. A similar slowing of this electron-transfer step was noted in D61A cells (see above).

(ii) Charge Recombination between Q_A^- and PSII Electron Donors. The decay of fluorescence yield that followed a saturating flash given to E65Q, E65A, and H92L cells in the presence of DCMU is shown in Figure 6A−C. The data for E65D cells (not shown) were similar to those for E65Q cells except that the maximal $[(F - F_{eq})/(F_{max} - F_{eq})]$ ratio was higher (the ratio was approximately 0.93 in E65D cells). The amplitudes and rates of the decay components in all four mutants are presented in Table 1. None of the Glu-65 mutants exhibited significant amounts of rapid decay kinetics that could correspond to charge recombination between Q_A^- and Y_Z^{ox}. Furthermore, the kinetics of fluorescence decay were essentially the same in wild-type* and E65D cells and were only marginally faster in E65Q and E65A cells (Figure 6A,B, and Table 1). These results suggest that essentially all PSII reaction centers in E65D, E65Q, and E65A cells contain intact Mn clusters with S_2/S_1 midpoint potentials that are essentially the same as in wild-type* cells. In contrast, the charge recombination kinetics of H92L cells (Figure 6C) was more rapid and heterogeneous. Furthermore, a small fraction of reaction centers in this mutant may exhibit charge recombination between Q_A^- and Y_Z^{ox} (Table 1). These results suggest that the equilibrium concentration of P_{680}^+

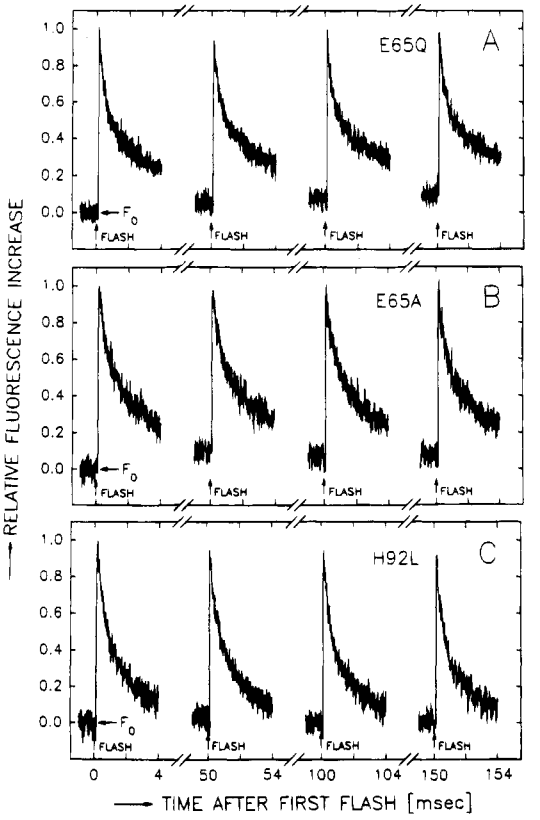

FIGURE 5: Yields of variable chlorophyll *a* fluorescence produced by each of four saturating flashes given at 50 ms intervals to Glu-65 and His-92 mutant cells. Five milliseconds of data are shown for each flash. (A) E65Q, (B) E65A, (C) H92L. The conditions were the same as in Figure 1. The maximum $(F - F_0)/F_0$ values measured after the first flash in each series were 0.41 for (A), 0.34 for (B), and 0.33 for (C). Data obtained with cells of E65D (not shown) resembled those of E65Q and E65A cells. Three individual flash series were averaged for (A), two for (B), and two for (C). The frequency of the monitoring flashes was switched from 1.6 to 100 kHz for 5 ms beginning 1 ms before each flash.

(and the S_2/S_1 midpoint potential) is higher in this mutant than in wild-type* cells. If all PSII reaction centers in this mutant contain fully functional Mn clusters (as suggested by the essentially wild-type rates of oxygen evolution exhibited by the PSII reaction centers in this mutant), the possible observation of some $Q_A^- \rightarrow Y_Z^{ox}$ charge recombination after a single flash given in the presence of DCMU (Figure 6C) suggests that electron transfer from the Mn cluster to Y_Z^{ox} may be slowed during the $S_1 \rightarrow S_2$ transition. However, the rate is not slowed enough to affect oxygen evolution or enough that significant amounts of Y_Z^{ox} remain oxidized 50 ms after a saturating flash given in the absence of DCMU (Figure 5C).

(iii) Photoaccumulation of Q_A^-. To confirm that insignificant fractions of PSII reaction centers in the H92L and Glu-65 mutants lack photooxidizable Mn ions *in vivo*, cells were illuminated for 1−15 s in the presence of DCMU. The kinetics of Q_A^- oxidation in E65Q, E65A, and H92L cells after 5 s of illumination are presented in Figure 6D−F. The data of E65D cells (not shown) resembled those of E65Q cells. In all three Glu-65 mutants, the fractions of Q_A^- that photoaccumulated during 5 s of illumination (8.5−9.5%, Figure 6D,E) were similar to that in wild-type cells (7.2% ± 0.9%, Figure 2E). Furthermore, the rates of Q_A^- photoaccumulation were essentially the same as that in wild-type*

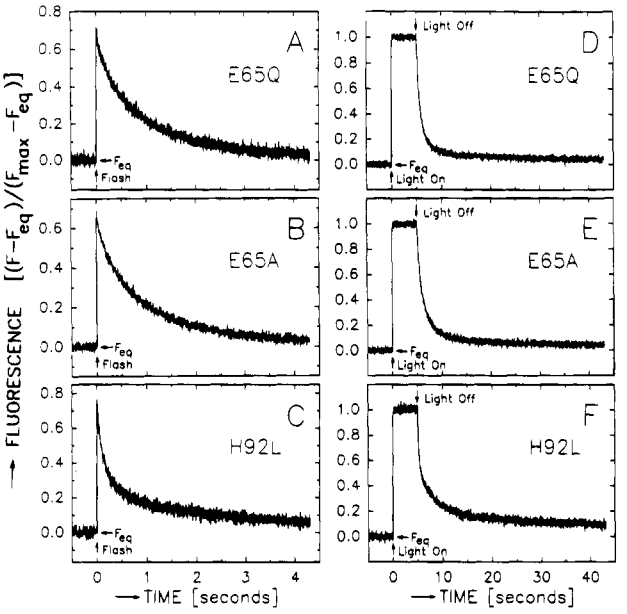

FIGURE 6: Formation and decay of Q_A^- in response to a saturating flash (left panels) or to 5 s of illumination (right panels) given to Glu-65 and His-92 mutant cells in the presence of DCMU, as measured by changes in the yield of chlorophyll *a* fluorescence: (A, D) E65Q; (B, E) E65A; (C, F) H92L. Note the 10-fold difference in time scale between the left and right panels. The conditions were the same as in Figure 2. Traces A−C represent the computer averages of 5, 11, and 8 traces, respectively. The monitoring flashes were applied at 1.6 kHz. The data of E65D cells (not shown) resembled those of E65Q cells.

cells (Figure 3B). These data confirm that essentially all reaction centers in all three Glu-65 mutants contain photooxidizable Mn clusters. In contrast, in H92L cells, the fraction of Q_A^- that photoaccumulated during 5 s of illumination (16% ± 3%, Figure 6F) was higher than that in wild-type* cells, and the rate of Q_A^- photoaccumulation was greater (Figure 3B). However, little or no Q_A^- photoaccumulated during the first second of illumination. These data confirm that essentially all reaction centers in H92L cells contain photooxidizable Mn clusters. The more rapid rate of Q_A^- photoaccumulation is consistent with the higher equilibrium concentration of P_{680}^+ inferred to be present in this mutant (see above).

(iv) Flash-Induced Increases in Fluorescence Yield. In all four mutants, the increase in fluorescence yield produced by a saturating flash (relative to F_{max}) was slightly lower than that in wild-type* cells (compare Figure 6A−C with Figure 2A). To test the possibility that slowed electron transfer from Y_Z to P_{680}^+ contributed to the lower flash-induced increases, the yield produced by the first in a series of saturating flashes was measured in the presence of DCMU and hydroxylamine. In all four mutants, the ratio $(F_1 - F_0)/(F_{max} - F_0)$ was similar to that in wild-type* cells. [The ratios were 0.66 ± 0.04 for E65D cells, 0.61 ± 0.04 for E65Q cells, 0.61 ± 0.07 for E65A cells, and 0.81 ± 0.03 for H92L cells, compared to 0.59 ± 0.06 for wild-type* cells (data not shown).] Consequently, the lower flash-induced fluorescence increases observed in these mutants in the *absence* of hydroxylamine (Figure 6A−C) were not caused by slowed electron transfer from Y_Z to P_{680}^+, unless this electron-transfer step is slowed substantially (relative to wild type) only in the *absence* of hydroxylamine. In H92L cells, the lower flash-induced increase in the absence of hydroxylamine (Figure 6C) correlated with this mutant's accelerated $S_2 \rightarrow Q_A^-$ charge recombination kinetics. Consequently, the lower flash-induced increase in the H92L mutant may be caused by the higher equilibrium concentration of P_{680}^+ inferred to be present (see above). This correlation does not apply for the E65Q and E65A mutants, however (Figure 6A,B). Consequently, the lower flash-induced increases observed in these mutants in the *absence* of hydroxylamine may be caused, in part, by the presence of a fluorescence quencher other than P_{680}^+, as concluded earlier for the D61A mutant.

(C) Involvement in Mn Binding. From the results presented earlier, we conclude that neither Glu-65 nor His-92 is essential for the assembly or stability of the Mn cluster. Therefore, it is unlikely that either residue ligates Mn. The slow kinetics of charge recombination and rates of Q_A^- photoaccumulation in E65D, E65Q, E65A, and H92L cells (Figures 6 and 3B) show that these mutants contain few, if any, PSII reaction centers that lack photooxidizable Mn ions. The absence of apparent fluorescence quenching following the second and subsequent flashes given in the absence of DCMU (Figure 5) implies that none of the S-state transitions are slowed dramatically in these mutants. However, the lower light-saturated rates of oxygen evolution in E65Q and E65A cells (Table 1) imply that at least one S-state transition or O_2 release is slowed to some extent in these mutants, as concluded earlier for the D59N mutant.

(D) Involvement in Ca^{2+} Binding. Because all three Glu-65 mutants grew photoautotrophically in media without Ca^{2+} ions, we conclude that Glu-65 has no influence on Ca^{2+} binding in PSII. However, as noted earlier, H92L cells exhibited little or no photoautotrophic growth in medium containing Na^+ substituted for Ca^{2+}. The light-saturated oxygen evolution rate of H92L cells propagated photoheterotrophically under such conditions was less than 5% compared to that of wild-type* cells (not shown). Unlike D59N and D61A cells, H92L cells exhibited no unusual light sensitivity when propagated under these conditions, that is, they exhibited normal flash-induced increases in fluorescence yield in the absence of Ca^{2+} (not shown). However, the kinetics of charge recombination after a single flash given in the presence of DCMU was slowed considerably (not shown), resembling that of cells lacking the extrinsic 33 kDa polypeptide (Chu et al., 1994b). Because the effects of propagating H92L cells in medium having Na^+ substituted for Ca^{2+} were much lower compared to D59N and D61A cells, and because H92L cells grew photoautotrophically in the presence of Mg^{2+} or Sr^{2+}, we conclude that the H92L mutation causes structural changes that are sufficient to weaken the affinity of a Ca^{2+} site for Ca^{2+} ions.

(III) Asp-170

(A) Growth, Oxygen Evolution Characteristics, and PSII Contents of Mutants. Numerous mutations at this position have been studied previously (Nixon & Diner, 1992; Boerner et al., 1992; Diner & Nixon, 1992; Whitelegge et al., 1992, 1995; Chu et al., 1994a). However, the essentially normal S_2-state lifetime reported for the D170R mutant (Nixon & Diner, 1992) and our isolation of a photoautotrophic pseudorevertant of D170A cells that had Val substituted for Ala-170 (unpublished result) led us to construct the additional mutants D170V, D170L, and D170I. The D170V mutant

was photoautotrophic, but grew slowly in the absence of glucose, with an A_{730} doubling time of 30−40 h compared to 13−18 h for wild-type* cells (data not shown). Both D170L and D170I cells required glucose for propagation. All three mutants evolved oxygen, but at diminished rates compared to wild-type* cells (Table 1). The oxygen evolution exhibited by these mutants was so surprising to us that we constructed each mutant on two separate occasions. Not one lost significant O_2-evolving activity when illuminated with saturating light. The apparent PSII contents of the D170L and D170I mutants were similar to that of wild-type* cells, while that of D170V cells was significantly less (approximately 77% compared to wild-type*, see Table 1). Taking into account the apparent PSII contents, the data of Table 1 show that the PSII reaction centers in D170V cells evolved oxygen at approximately 50% of the rate of wild-type* reaction centers, while those in D170L and D170I cells evolved oxygen at 20−24% of the wild-type* rate. [In comparison, D170E, D170H, and D170R reaction centers evolved oxygen at approximately 60%, 50%, and 13% of the rate of wild-type* reaction centers, respectively (Chu et al., 1994a).] All three mutants exhibited O_2 yields that oscillated with a period of 4 in response to a series of saturating flashes, showing that the oxygen released originated from functional Mn clusters [these and other O_2 measurements (not shown) were kindly performed for us by R. L. Burnap (Oklahoma State University) and will be presented elsewhere].

(B) Fluorescence Characteristics of Mutants. (i) Electron Transfer to Y_Z^{ox}. The fluorescence yields that followed each of four saturating flashes spaced 50 ms apart are shown in Figure 7. In all three mutants, the maximum fluorescence yield after the second and subsequent flashes was only partly quenched compared to mutants such as D170A, D170T, and D170N, mutants that contain no photooxidizable Mn ions (Chu et al., 1994a). Indeed, the data of D170V, D170L, and D170I cells (Figure 7A−C, respectively) resemble those of D170H and D170R cells, which contain significant fractions of PSII reaction centers *with* photooxidizable Mn ions [estimated to be approximately 60% and 40% in D170H and D170R cells, respectively (Chu et al., 1994a)].

In each mutant, some Q_A^- remained reduced 50 ms after each flash. The amount decreased as the spacing between flashes increased (not shown), showing that the slowest components of electron transfer from Q_A^- to Q_B were slowed in all three mutants. A similar slowing was previously noted in D61A, E65Q, and E65A cells (see above) and in other Asp-170 mutants (Chu et al., 1994a).

(ii) Charge Recombination between Q_A^- and PSII Electron Donors. The decay of fluorescence yield that followed a saturating flash given to D170V, D170L, and D170I cells in the presence of DCMU is shown in Figure 8A−C. The amplitudes and rates of the decay components are presented in Table 1. All three mutants exhibited decay kinetics that resembled that of D170H cells (Chu et al., 1994a), that is, all three exhibited significant amounts of rapid decay kinetics, which corresponds to charge recombination between Q_A^- and Y_Z^{ox} plus slower decay components that could correspond to charge recombination between Q_A^- and the S_2 state of the Mn cluster (Table 1). In all three mutants, the rates of the slower decay components were similar to that

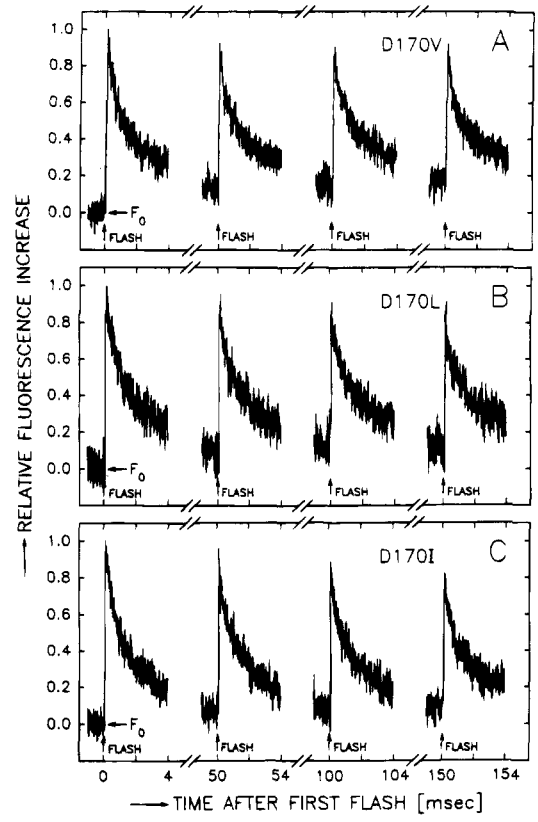

FIGURE 7: Yields of variable chlorophyll *a* fluorescence produced by each of four saturating flashes given at 50 ms intervals to Asp-170 mutant cells. Five milliseconds of data are shown for each flash. (A) D170V, (B) D170L, (C) D170I. The conditions were the same as in Figure 1. The maximum $(F - F_0)/F_0$ values measured after the first flash in each series were 0.20 for (A), 0.16 for (B), and 0.17 for (C). Five individual flash series were averaged for (A), five for (B), and four for (C). The frequency of the monitoring flashes was switched from 1.6 to 100 kHz for 5 ms beginning 1 ms before each flash.

in wild-type* cells (Table 1). These results suggest that the Mn clusters in the D170V, D170L, and D170I mutants have S_2/S_1 midpoint potentials that differ little from that of wild type.

(iii) Photoaccumulation of Q_A^-. To estimate the fractions of PSII reaction centers in D170V, D170L, and D170I cells that contained photooxidizable Mn ions, cells were illuminated for 1−15 s in the presence of DCMU. The fractions of Q_A^- that photoaccumulated during 5 s of illumination were 43% ± 7%, 49% ± 5%, and 50% ± 6% in D170V, D170L, and D170I cells, respectively (Figure 8D−F), compared to 7.2% ± 0.9% in wild-type* cells (Figure 2E). In all three mutants, the rates of Q_A^- photoaccumulation were heterogeneous, with a fraction of reaction centers photoaccumulating Q_A^- during the first second of illumination and other fractions photoaccumulating with rates similar to that in wild-type* cells (Figure 9A). By comparing the extent of Q_A^- photoaccumulation in D170V, D170L, and D170I cells after 10−15 s of illumination with that in wild-type* and D170A cells (Figure 9A), we estimate that only about 50% of D170V reaction centers and 60% of D170L and D170I reaction centers *lack* photooxidizable Mn clusters *in vivo*. These estimates are in rough agreement with the fractions of Q_A^- that appeared to recombine with Y_Z^{ox} after a single saturating flash given in the presence of DCMU (Figure 8A−C, Table 1). However, these estimates should

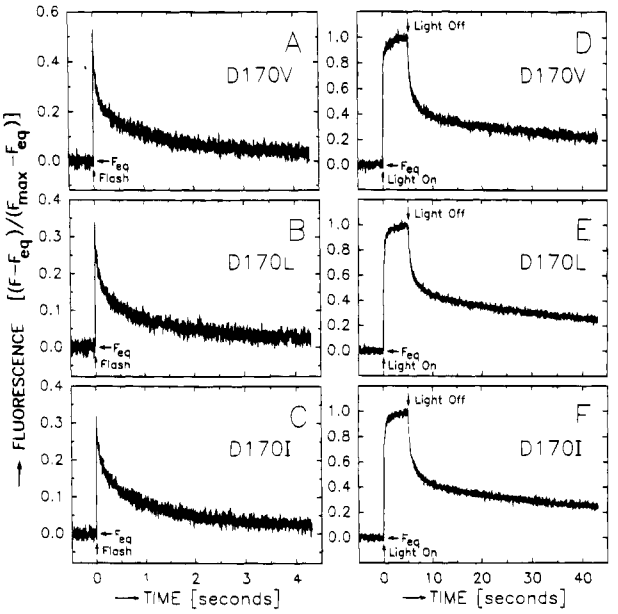

FIGURE 8: Formation and decay of Q_A^- in response to a saturating flash (left panels) or to 5 s of illumination (right panels) given to Asp-170 mutant cells in the presence of DCMU, as measured by changes in the yield of chlorophyll *a* fluorescence: (A, D) D170V; (B, E) D170L; (C, F) D170I. Note the 10-fold difference in time scale between the left and right panels. The conditions were the same as in Figure 2. Traces A−C represent the computer averages of 9, 12, and 16 traces, respectively. The monitoring flashes were applied at 1.6 kHz.

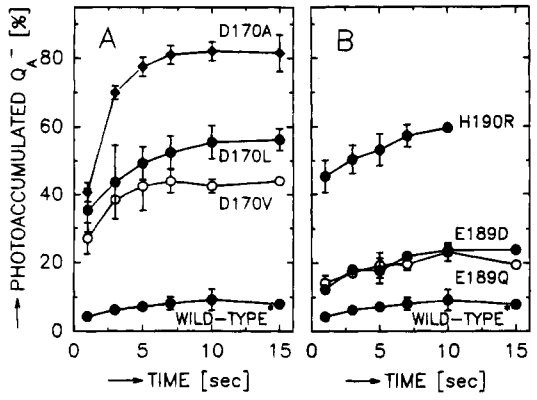

FIGURE 9: Fraction of PSII reaction centers in Asp-170, Glu-189, and His-190 mutant cells that photoaccumulated Q_A^- after illumination for specific intervals of time (*e.g.*, see Figure 8D−F and text): (A) photoaccumulation of Q_A^- in D170V and D170L cells in comparison to wild-type* and D170A cells (the data of D170I cells (not shown) were similar to those of D170L cells); (B) photoaccumulation of Q_A^- in E189Q, E189D, and H190R cells in comparison to wild-type* cells (the data of E189N cells (not shown) resembled those of E189Q and E189D cells). The wild-type* and D170A data are taken from Chu et al. (1994a) to facilitate comparisons. The conditions were the same as in Figure 3.

be considered to be very approximate for reasons enumerated elsewhere (Chu et al., 1994a).

(iv) Flash-Induced Increases in Fluorescence Yield. In all three mutants, the increase in fluorescence yield produced by a saturating flash (relative to F_{max}) was significantly lower than that in wild-type* cells, particularly for the D170L and D170I mutants (compare Figure 8A−C with Figure 2A). Reaction centers without photooxidizable Mn ions presumably contribute to the lowered yields because of the higher equilibrium concentrations of P_{680}^+ present in such reaction centers. However, when all three mutants were illuminated with a series of flashes in the presence of DCMU and hydroxylamine (not shown), the ratio $(F_1 - F_0)/(F_{max} - F_0)$ was lower than that in wild-type* cells [the ratios were 0.53 ± 0.02 for D170V cells, 0.43 ± 0.03 for D170L cells, and 0.36 ± 0.04 for D170I cells, compared to 0.59 ± 0.06 for wild-type* cells]. Consequently, for all three mutants (with the possible exception of D170V), slowed electron transfer from Y_Z to P_{680}^+ must contribute to the lower flash-induced increases in fluorescence yield in the *absence* of hydroxylamine. Lower $(F_1 - F_0)/(F_{max} - F_0)$ ratios were observed previously in most other Asp-170 mutants (Chu et al., 1994a). The lower $(F_1 - F_0)/(F_{max} - F_0)$ ratios in D170L and D170I cells (and possibly in D170V cells) suggest that these mutations, like most Asp-170 mutations previously characterized (Chu et al., 1994a), structurally perturb PSII to some degree.

(C) Involvement in Mn Binding. Although there are examples of Arg possibly replacing Met (Hampsey et al., 1986) or His (Sorrell et al., 1989; Garcia et al., 1992) as a ligand to Fe in cytochrome *c*, it seems inconceivable that Val, Leu, or Ile could replace Asp-170 as a ligand to Mn. Furthermore, it seems surprising that the Asp-170 → Arg mutation would have so little impact on the properties of the O_2-evolving reaction centers in D170R cells (Nixon & Diner, 1992), if Asp-170 ligates the Mn cluster. One explanation is that Asp-170, although required for efficient photooxidation of the first Mn ion ligated during the assembly of the Mn cluster, does not remain as a ligand to the final, *assembled* Mn cluster (Tang et al., 1994b). However, if Asp-170 is not a ligand to the *assembled* Mn cluster, it is difficult to understand why the replacement of Asp-170 by the isosteric residue Asn is more deleterious to oxygen evolution (Nixon & Diner, 1992; Boerner et al., 1992; Chu et al., 1994a) than the replacement of Asp-170 by Val, Leu, or Ile. An alternate explanation is that Val, Leu, and Ile, being bulky hydrophobic residues, cause structural perturbations that permit the missing carboxylate moiety to be replaced by another residue, a peptide carbonyl oxygen, or a water molecule. A similar compensatory structural change may occur in the D170R mutant. Subtle structural alterations have been inferred to occur in most Asp-170 mutants because of the smaller $(F_1 - F_0)/(F_{max} - F_0)$ ratios observed when these mutants are illuminated with a series of flashes in the presence of DCMU and hydroxylamine [see above and Chu et al. (1994a)]. In addition, the unusually rapid $Q_A^- \rightarrow Y_Z^{ox}$ charge recombination kinetics observed in D170S (Nixon & Diner, 1992) and D170A (Chu et al., 1994a) cells suggest that structural changes affecting the Y_Z^{ox}/Y_Z midpoint potential are caused by the replacement of Asp-170 with a much smaller residue.

Compensatory, mutation-induced structural rearrangements have been observed in other systems. In ferredoxin I of *Azotobacter vinelandii*, the replacement of Cys-20 with Ala caused a structural rearrangement that permitted the free Cys-24 residue to replace Cys-20 as a ligand to the [4Fe-4S] cluster (Martín et al., 1990). In ricin A, replacement of the active site residue Glu-177 with Ala resulted in the rotation of Glu-208 into the active site with partial restoration of catalytic activity (Kim et al., 1992). In human alcohol dehydrogenase, replacement of the pyrophosphate binding residue Arg-47 with Gly resulted in structural alterations that moved Lys-228 into the pyrophosphate site with partial restoration of activity (Stone et al., 1993). Finally, imidazole

and other exogenous organic substances can replace His-117 as a ligand to Cu in the H117G mutant of azurin from *Pseudomonas aeruginosa* (den Blaauwen & Canters, 1993) and His-93 as a ligand to Fe in the H93G mutant of sperm whale myoglobin (Barrick, 1994; DePillis et al., 1994).

On the basis of the preceding considerations, we conclude that the oxygen-evolving characteristics of D170V, D170L, and D170I cells do not eliminate Asp-170 as a possible ligand of the Mn cluster in PSII.

(D) Involvement in Ca^{2+} Binding. The Ca^{2+} requirements of D170H and D170A cells (Chu et al., 1994a) were investigated. The D170H mutant grew photoautotrophically at normal rates when Ca^{2+} was omitted from the growth medium, and the light-saturated rate of oxygen evolution decreased only slightly, from 46% ± 11% (Chu et al., 1994a) to approximately 30% (not shown). In addition, the fluorescence properties of these cells were essentially unchanged when propagated photoheterotrophically in medium having Na^+ substituted for Ca^{2+}. However, in D170A cells propagated in such media, the $(F_1 - F_0)/(F_{max} - F_0)$ ratio decreased from 0.43 ± 0.03 (Chu et al., 1994a) to 0.29 ± 0.02 (not shown). Nevertheless, because of the relatively small alterations in the properties of D170H and D170A cells in comparison to those of D59N, D61A, and H92L cells when propagated in the absence of Ca^{2+} ions (see above), we conclude that Asp-170 has little or no influence on Ca^{2+} binding in PSII.

(IV) Glu-189

(A) Growth, Oxygen Evolution Characteristics, and PSII Contents of Mutants. The following mutants were constructed: E189D, E189Q, and E189N. Only the E189Q mutant was photoautotrophic; it grew at normal rates. The E189D and E189N cells required glucose for propagation. The photoautotrophic growth of E189Q cells was impaired slightly when Ca^{2+} was replaced by Na^+: the A_{730} doubling time increased from 14−17 h in the presence of Ca^{2+} to approximately 21 h in its absence. The E189Q mutant exhibited a slightly impaired light-saturated oxygen evolution rate (Table 1), but it did not lose activity when illuminated with saturating light. In contrast, the E189D mutant evolved no oxygen, and the E189N mutant evolved oxygen at only 6% ± 2% of the rate of wild-type* cells (Table 1). The low rate of oxygen evolution in E189N cells was not caused by populations of reverted cells in the cultures. This was determined by spreading duplicate serial dilutions of cultures on solid media containing and lacking glucose (Chu et al., 1994a). None of the Glu-189 mutants had significantly depressed apparent PSII contents compared to wild-type* cells (Table 1). Consequently, the PSII reaction centers in E189Q cells evolved oxygen at about 70% the rate of wild-type* reaction centers.

(B) Fluorescence Characteristics of Mutants. (i) Electron Transfer to Y_Z^{ox}. The fluorescence yields that followed each of four saturating flashes spaced 50 ms apart are shown in Figure 10 for the mutants E189Q, E189D, and E189N. In E189Q cells (Figure 10A), the maximum fluorescence yield after each flash was essentially the same. In contrast, in E189D and E189N cells (Figure 10B,C), the maximum fluorescence yield after the second and subsequent flashes was quenched substantially. Indeed, the progressive quenching observed in the E189D mutant resembled that in mutants

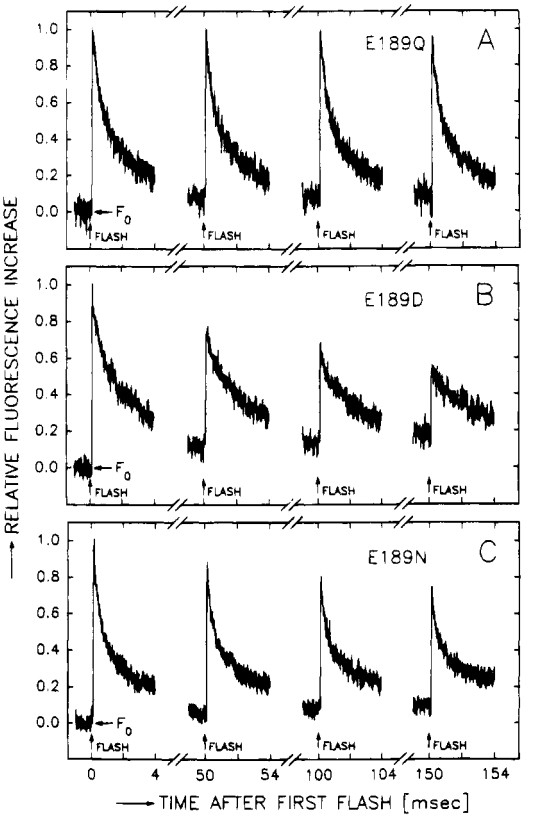

FIGURE 10: Yields of variable chlorophyll *a* fluorescence produced by each of four saturating flashes given at 50 ms intervals to Glu-189 mutant cells. Five milliseconds of data are shown for each flash. (A) E189Q, (B) E189Q, (C) E189N. The conditions were the same as in Figure 1. The maximum $(F - F_0)/F_0$ values measured after the first flash in each series were 0.23 for (A), 0.17 for (B), and 0.37 for (C). Five individual flash series were averaged for (A), eight for (B), and six for (C). The frequency of the monitoring flashes was switched from 1.6 to 100 kHz for 5 ms beginning 1 ms before each flash.

that lack photooxidizable Mn ions [*e.g.*, in the mutants D170A, D170N, and D170T (Chu et al., 1994a)]. In the E189D and E189N mutants, the extent of quenching diminished as the spacing between saturating flashes was increased, becoming negligible at flash spacings of 600−800 ms (not shown).

In all three mutants, some Q_A^- remained reduced 50 ms after each flash. The amount decreased as the spacing between flashes increased (not shown), showing that the slowest components of electron transfer from Q_A^- to Q_B were slowed, particularly in E189D cells. A similar slowing was noted earlier in other mutants (see above).

(ii) Charge Recombination between Q_A^- and PSII Electron Donors. The decay of fluorescence yield that followed a saturating flash given to E189Q and E189D cells in the presence of DCMU is shown in Figure 11A,B. The data of E189N cells (not shown) resembled those of E189D cells. The amplitudes and rates of the decay components are presented in Table 1. All three mutants exhibited decay kinetics that closely resembled that of wild-type* cells (Figure 2A). These results suggest that nearly all PSII reaction centers in these mutants contain intact Mn clusters with S_2/S_1 midpoint potentials that are essentially the same as that in wild-type* cells. However, an alternate explanation is that the midpoint potential of Y_Z^{ox}/Y_Z is altered sufficiently in the E189D and E189N mutants that the observed charge

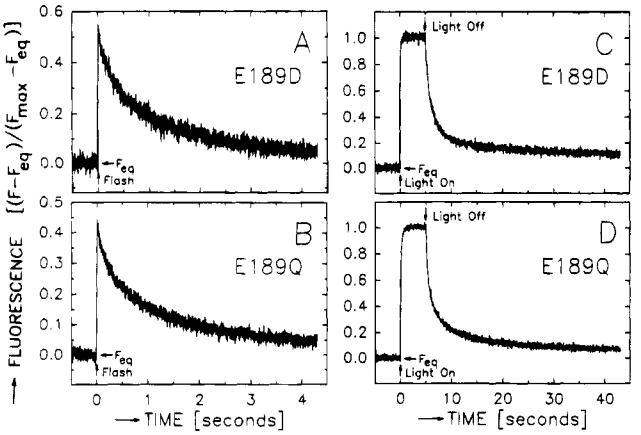

FIGURE 11: Formation and decay of Q_A^- in response to a saturating flash (left panels) or to 5 s of illumination (right panels) given to Glu-189 mutant cells in the presence of DCMU, as measured by changes in the yield of chlorophyll *a* fluorescence: (A, C) E189D; (B, D) E189Q. Note the 10-fold difference in time scale between the left and right panels. The conditions were the same as in Figure 2. Traces A and B represent the computer averages of 8 and 13 traces, respectively. The monitoring flashes were applied at 1.6 kHz. The data of E189N cells (not shown) resembled those of E189D cells.

recombination kinetics corresponds to slow recombination between Q_A^- and Y_Z^{ox}. To test this possibility, the Δ*psb*O gene that encodes the extrinsic 33 kDa polypeptide was deleted from the mutants E189Q and E189D. (We reasoned that $Q_A^- \rightarrow Y_Z^{ox}$ and $Q_A^- \rightarrow S_2$ recombinations should be altered differently in the absence of this polypeptide.) In *both* mutants, the kinetics of charge recombination after a saturating flash given in the presence of DCMU slowed considerably in the absence of the extrinsic 33 kDa polypeptide (not shown), resembling those of other mutants without this polypeptide (Chu et al., 1994b). Because the kinetics was altered to the *same extent* in both E189Q and E189D cells, we conclude that the slow charge recombination kinetics of E189D cells in the *presence* of the extrinsic 33 kDa polypeptide (Figure 11A) corresponds to charge recombination between Q_A^- and the Mn cluster. As an additional test, E189D cells were propagated in medium having Ca^{2+} replaced by Sr^{2+}. The charge recombination kinetics of these cells (not shown) resembled those of D170H and D170V cells (see Figure 8A), that is, Q_A^- appeared to recombine with Y_Z^{ox} in some reaction centers and with the S_2 state of the Mn cluster in the remainder. We conclude that the charge recombination kinetics of E189D and E189N cells correspond to recombination between Q_A^- and the S_2 state of the Mn cluster.

(iii) Photoaccumulation of Q_A^-. To estimate the fractions of PSII reaction centers in E189Q, E189D, and E189N cells that lack photooxidizable Mn ions, cells were illuminated for 1−15 s in the presence of DCMU. The fractions of Q_A^- that photoaccumulated during 5 s of illumination were 18% ± 4%, 19% ± 2%, and 19% ± 4% in E189Q, E189D, and E189N cells, respectively (Figure 11C,D), compared to 7.2% ± 0.9% in wild-type* cells (Figure 2E). Surprisingly, approximately the same fraction of Q_A^- photoaccumulated during the first second of illumination in all three mutants (Figure 9B). The remaining fractions photoaccumulated with rates similar to that in wild-type* cells. By comparing the extent of Q_A^- photoaccumulation in these cells after 10−15 s of illumination with that in wild-type* and D170A cells (Figure 9A), we estimate that only 12−14% of E189Q, E189D, and E189N reaction centers lack photooxidizable Mn clusters *in vivo*.

(iv) Flash-Induced Increases in Fluorescence Yield. In all three mutants, the increase in fluorescence yield produced by a saturating flash (relative to F_{max}) was significantly lower than that in wild-type* cells (compare Figure 11A,B with Figure 2A). However, when these mutants were illuminated with a series of flashes in the presence of DCMU and hydroxylamine (not shown), the ratio $(F_1 - F_0)/(F_{max} - F_0)$ was substantially lower than that in wild-type* cells for only the E189N mutant [the ratios were 0.52 ± 0.04 for E189Q cells, 0.58 ± 0.02 for E189D cells, and 0.36 ± 0.03 for E189N cells, compared to 0.59 ± 0.06 for wild-type* cells]. Consequently, in the E189N mutant, slowed electron transfer from Y_Z to P_{680}^+ must contribute to the lower flash-induced increase in fluorescence yield in the *absence* of hydroxylamine. The charge recombination kinetics of the E189Q, E189D, and E189N mutants give little indication that the equilibrium concentration of P_{680}^+ is greater in these mutants than in wild-type* cells (Figure 11A,B). Consequently, the lower flash-induced increases in E189Q and E189D cells may be caused, in part, by the presence of a fluorescence quencher other than P_{680}^+, as concluded earlier for the D61A, E65Q, and E65A mutants.

(C) Involvement in Mn Binding. From the results presented earlier, we conclude that Glu-189 influences the assembly and/or stability of the Mn cluster. However, this influence is relatively minor because only 12−14% of the PSII reaction centers in the E189Q, E189D, and E189N mutants appear to lack photooxidizable Mn ions *in vivo*. On the basis of these results, and because the Glu → Gln mutation causes only minor perturbations in oxygen evolution compared to the Glu → Asp mutation, we conclude that Glu-189 is unlikely to ligate Mn. Nevertheless, the progressive quenching of the maximum yield of fluorescence following the second and subsequent flashes in E189D and E189N cells (Figure 10B,C) indicates that electron transfer from the Mn cluster to Y_Z^{ox} in these two mutants must be slowed substantially during one or more of the S-state transitions. That substantial quenching is evident following the second flash might suggest that electron transfer from Mn to Y_Z^{ox} is slowed during the $S_1 \rightarrow S_2$ transition. However, because the cells were not extensively dark-adapted, and because the weak monitoring flashes were applied at 100 kHz for 5 ms surrounding each saturating flash, it seems likely that, in many PSII reaction centers, the Mn cluster would have been in the S_2 or S_3 state prior to the firing of the first saturating flash. Indeed, because little or no $Q_A^- \rightarrow Y_Z^{ox}$ charge recombination was observed in these mutants after a single flash given in the presence of DCMU (Figure 11B and Table 1), we suggest that electron transfer from Mn to Y_Z^{ox} is slowed substantially during either the $S_2 \rightarrow S_3$ or $S_3 \rightarrow (S_4) \rightarrow S_0$ transition, rather than during the $S_1 \rightarrow S_2$ transition. As noted earlier in connection with the D61N and D61A mutants, it should be possible to test this assertion with O_2 flash yield experiments. The lower light-saturated rate of oxygen evolution in E189Q cells indicates that either the rate of O_2 release or the rate of electron transfer from Mn to Y_Z^{ox} is slowed to some extent during at least one S-state transition in this mutant. However, the rate is not slowed enough that significant amounts of Y_Z^{ox} remain oxidized 50

ms after a saturating flash given in the absence of DCMU (Figure 10A).

(D) Involvement in Ca^{2+} Binding. As mentioned earlier, E189Q cells grew photoautotrophically in the absence of Ca^{2+} ions, but at a slightly diminished rate. In addition, both the apparent PSII content and the oxygen evolution rate of E189Q cells were diminished slightly in the absence of Ca^{2+} ions [under these conditions, the apparent PSII content and oxygen evolution rate were approximately 60% and 25%, respectively, compared to wild-type* cells (not shown)]. However, the fluorescence properties of neither these nor E189D cells were altered appreciably when propagated in the absence of Ca^{2+} ions (not shown). In contrast, E189N cells propagated in the absence of Ca^{2+} exhibited a substantially diminished $(F_1 - F_0)/(F_{max} - F_0)$ ratio (less than 0.1) when illuminated with a series of flashes in the presence of DCMU and hydroxylamine (not shown). The relatively minor perturbations caused by propagating E189Q and E189D cells in the absence of Ca^{2+} imply that Glu-189 is unlikely to ligate a Ca^{2+} ion in PSII. The more severe consequences of propagating E189N cells in the absence of Ca^{2+} may result from the greater the structural perturbations inferred to be present in this mutant from the lower $(F_1 - F_0)/(F_{max} - F_0)$ ratio measured in the *presence* of Ca^{2+} ions (see above).

(E) A Possible Structural Role. The dramatic differences in the oxygen-evolving and fluorescence characteristics of E189Q and E189D cells suggest that Glu-189 performs a crucial structural role in optimizing water oxidation. We propose that Glu-189 participates in a network of hydrogen bonds and that this network is substantially maintained by Gln, but not by the smaller Asp and Asn residues. In dihydrofolate reductase, the hydrogen bond network involving Asp-27 is maintained in the D27N mutation: an O−HN hydrogen bond is merely replaced by an NH−N hydrogen bond (Howell et al., 1986). Because the extra methylene group increases the length and flexibility of Glu (and Gln) relative to Asp (and Asn) [*e.g.*, see Richardson and Richardson (1989)], the substitution of Glu by Asp is not always a conservative mutation. Indeed, there are several examples where a Glu → Asp substitution is deleterious to function because the position and orientation of the carboxylate moiety have changed in the structure of the mutant protein [*e.g.*, the E43D mutation in staphylococcal nuclease (Loll & Lattman, 1990) and the E165D mutation in triosephosphate isomerase (Joseph-McCarthy et al., 1994)]. Furthermore, there are examples where the Asp → Glu mutation is far more deleterious than the Asp → Asn mutation [*e.g.*, see Babu et al. (1992) and Mizrahi et al. (1994)]. In PSII, one possibility is that the proposed network of hydrogen bonds involving Glu-189 is crucial for positioning a residue that participates in proton release during water oxidation. The improper positioning of this residue in the E189D and E189N mutants could render electron transfer from Mn to Y_Z^{ox} thermodynamically unfavorable during, for example, the $S_2 \rightarrow S_3$ transition [*e.g.*, see Lavergne and Junge (1993)], thereby slowing this electron-transfer step and causing the progressive quenching in maximal fluorescence yield shown in Figure 10B,C.

(V) His-190

(A) Growth, Oxygen Evolution Characteristics, and PSII Contents of Mutants. The following mutants were constructed: H190R, H190N, H190Q, H190L, and H190Y. All five mutants required glucose for propagation. Only H190R cells evolved oxygen (at 13% ± 2% of the rate of wild-type* cells). This oxygen-evolving activity was rapidly lost in saturating light. The apparent PSII contents of H190R, H190N, and H190Q cells were similar to that of wild-type* cells, but those of H190L and H190Y cells were only 50−60% compared to wild-type* (Table 1).

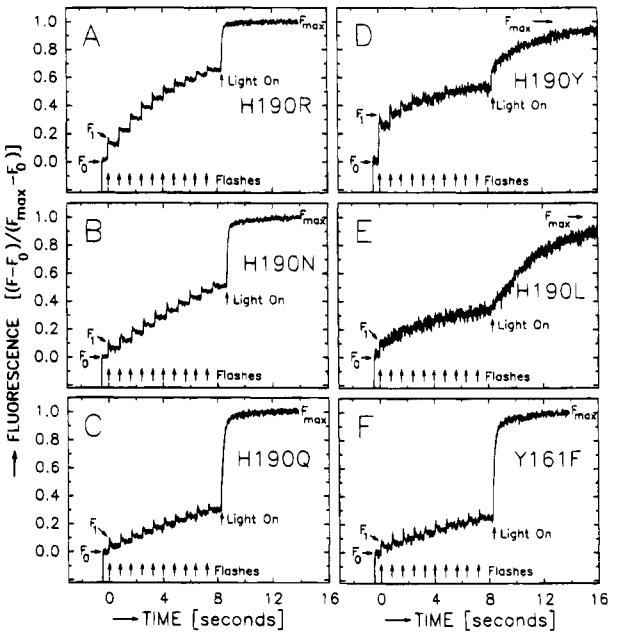

FIGURE 12: Formation of Q_A^- in Y161F and His-190 mutant cells in response to 10 saturating flashes given at 800 ms intervals in the presence of DCMU and hydroxylamine, followed by continuous illumination: (A) H190R, (B) H190N, (C) H190Q, (D) H190Y, (E), H190L, (F) Y161F. The conditions were the same as in Figure 4 except that the cells were all propagated at 50−60 μE m^{-2} s^{-1} in the presence of Ca^{2+} ions. The saturating flashes and the onset of continuous illumination are indicated by vertical arrows. The initial fluorescence yields produced by the monitoring flashes (F_0) and the maximal yields produced by continuous illumination (F_{max}) are also indicated. The monitoring flashes were applied at 1.6 kHz.

(B) Fluorescence Characteristics of Mutants. (i) Flash-Induced Increases in Fluorescence Yield. When illuminated with a series of flashes in the presence of DCMU and hydroxylamine, the $(F_1 - F_0)/(F_{max} - F_0)$ ratios were quite low in all five mutants. These ratios were 0.17 ± 0.01 for H190R cells (Figure 12A), 0.12 ± 0.02 for H190N cells (Figure 12B), 0.082 ± 0.016 for H190Q cells (Figure 12C), 0.36 ± 0.03 for H190Y cells (Figure 12D), and 0.13 ± 0.01 for H190L cells (Figure 12E), compared to 0.59 ± 0.06 for wild-type* cells (Chu et al., 1994a). The ratio in H190Q cells was comparable to that observed in the Y161F mutant (Figure 12F), which lacks Y_Z (Debus et al., 1988; Metz et al., 1989). The low $(F_1 - F_0)/(F_{max} - F_0)$ ratios suggest that electron transfer from Y_Z to P_{680}^+ is slowed dramatically in the His-190 mutants. The same conclusion was reached recently by Sayre and co-workers for the H190F and H190Y mutants of *Chlamydomonas reinhardtii* (Roffey et al., 1994a,b). In preliminary accounts, Nixon, Diner, and co-workers have reported that electron transfer from Y_Z to P_{680}^+ is slowed by 200-fold in the H190Q and H190D mutants of *Synechocystis* 6803 (Diner et al., 1991; Nixon & Diner, 1994).

(ii) Charge Recombination between Q_A^- and PSII Electron Donors. The decay of fluorescence yield that followed

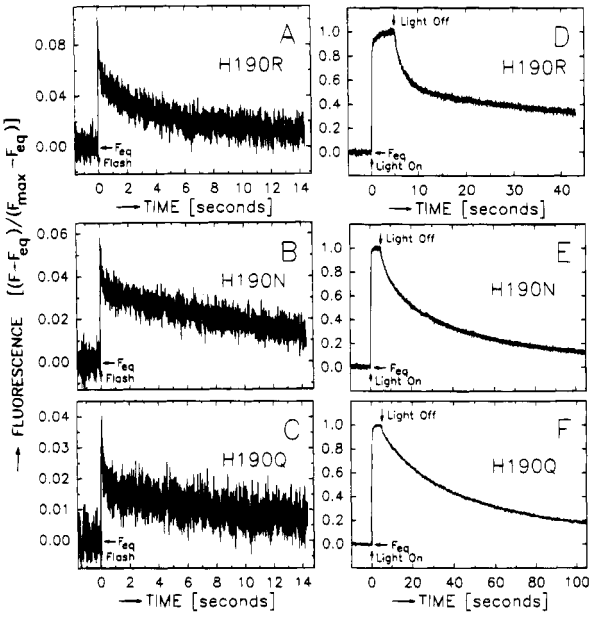

FIGURE 13: Formation and decay of Q_A^- in response to a saturating flash (left panels) or to 5 s of illumination (right panels) given to His-190 mutant cells in the presence of DCMU, as measured by changes in the yield of chlorophyll *a* fluorescence: (A, D) H190R; (B, E) H190N; (C, F) H190Q. Note the three different time scales in this figure. Also note that two of the time scales in this figure are considerably longer than those in Figures 2, 6, 8, and 11. The conditions were the same as in Figure 2. Traces A−C represent the computer averages of 18, 25, and 25 traces, respectively. The monitoring flashes were applied at 1.6 kHz.

a saturating flash given to H190R, H190N, and H190Q cells in the presence of DCMU is shown in Figure 13A−C. The H190L and H190Y mutants yielded data similar to those of the H190N and H190Q mutants, but with lower signal-to-noise ratios. Because the flash-induced increases in yield (relative to F_{max}) were quite low, the signal-to-noise ratios for the His-190 mutants were lower than those for other mutants. Consequently, in all but the H190R mutant, two exponential decay components were sufficient to yield random residuals. The amplitudes and rates of the decay components are presented in Table 1. In each mutant, 40−60% of the decay was relatively rapid, presumably corresponding to charge recombination between Q_A^- and Y_Z^{ox}. In all five mutants, the slowest components decayed very slowly, with inverse rate constants of 10−15 s (Table 1).

(iii) Photoaccumulation of Q_A^-. The decay of fluorescence yield that followed 5 s of illumination applied to H190R cells is presented in Figure 13D. In these cells, 56% ± 5% of Q_A^- photoaccumulated during 5 s of illumination. The fraction of Q_A^- that photoaccumulated during 1−15 s of illumination in H190R cells is shown in Figure 9B. The rate of photoaccumulation was heterogeneous, with a considerable fraction of reaction centers photoaccumulating Q_A^- during the first second of illumination and other fractions photoaccumulating at slower rates. These data (and the oxygen-evolving activity of these cells) imply that a significant fraction of PSII reaction centers in this mutant contain photooxidizable Mn ions *in vivo*. By comparing the extent of Q_A^- photoaccumulation after 5−10 s with that in wild-type* and D170A cells, we estimate that 30−33% of H190R reaction centers *contain* photooxidizable Mn ions *in vivo*.

The decay of fluorescence yield that followed 5 s of illumination applied to H190N and H190Q cells is presented in Figure 13E,F. Similar data were obtained even after 1 s of illumination: 80−90% of the decay in these mutants was characterized by inverse rate constants of 17−90 s (not shown). We suggest that an alternate donor reduces P_{680}^+ with a relatively high quantum yield in all His-190 mutants except H190R. Reduction of P_{680}^+ by such a donor would account for the slow Q_A^- oxidation kinetics observed both after a single flash (Figure 13B,C) and after short periods of illumination (Figure 13E,F) in the H190N and H190Q mutants in the presence of DCMU. A similar model involving the reduction of Y_Z^{ox} by an alternate donor has been proposed by Sayre and co-workers to explain similar data obtained with the H190F and H190Y mutants of *Chlamydomonas reinhardtii* (Roffey et al., 1994a). The identity of the alternate donor is unknown.

(C) Sensitivity to Light. When His-190 mutants were propagated in dim light (5−6 μE m^{-2} s^{-1}), their apparent PSII contents diminished and the oxygen evolution activity of H190R cells decreased, but the $(F_1 - F_0)/(F_{max} - F_0)$ ratios measured in the presence of DCMU and hydroxylamine increased (not shown). Similar results were described earlier for D59N and D61A cells propagated in the absence of Ca^{2+} ions (*e.g.*, Figure 1C,D). That the $(F_1 - F_0)/(F_{max} - F_0)$ ratios increased when the cells were propagated in dim light shows that the His-190 mutants are extremely light-sensitive. The H190F and H190Y mutants of *Chlamydomonas reinhardtii* have also been reported to be extremely light-sensitive (Roffey et al., 1994a,b). The EPR signals of Y_D^{ox} and Y_Z^{ox} were reported to be lost rapidly during illumination of the latter mutants (Roffey et al., 1994b). However, neither we (Bernard et al., 1995) nor others [X.-S. Tang, P. J. Nixon, and B. A. Diner, unpublished results cited in Tang et al. (1993a)] have encountered difficulties in generating these signals by continuous illumination of H190Q, H190D, or H190E preparations from *Synechocystis* 6803.

(D) Involvement in Mn Binding. The assembly and activation of the Mn cluster *in vivo* are characterized by a very low quantum yield (Cheniae & Martin, 1971a). Nevertheless, the oxygen evolution activity of H190R cells shows that oxygen-evolving Mn clusters can be assembled in these cells in spite of a low quantum yield for $Y_Z^{ox}Q_A^-$ formation (Figure 12A). Because the apparent quantum yield for $Y_Z^{ox}Q_A^-$ formation was not dramatically higher in H190R cells than in other His-190 mutants (Figure 12), additional factors must contribute to the absence of oxygen-evolving Mn clusters from the latter mutants. Because Arg can functionally replace a His ligand to Fe in cytochrome *c* (Sorrell et al., 1989; Garcia et al., 1992), one possibility is that His-190 is a ligand to the Mn cluster. The possible ligation of Mn by His-190 has been suggested previously by Nixon, Diner, and co-workers on the basis of examination of several *Synechocystis* His-190 mutants (Nixon & Diner, 1994; Tang et al., 1994a,b) and by Sayre and co-workers on the basis of examination of the H190F mutant of *Chlamydomonas reinhardtii* (Roffey et al., 1994a). However, existing models for the structure of the D1/D2 heterodimer (Svensson et al., 1990, 1991, 1992; Ruffle & Nugent, 1992; Ruffle et al., 1992) place His-190 and Y_Z in closer proximity than would be predicted on the basis of an EPR study that places the nearest Mn atom >10 Å from Y_Z (Hoganson & Babcock, 1988). An alternate possibility is that His-190 serves as a crucial base or as a crucial hydrogen bond donor

that can be partly replaced by Arg, but not by the smaller Gln and Asn residues.

SUMMARY AND CONCLUSIONS

Of all of the conserved carboxylate, histidine, and tyrosine residues in the lumenal interhelical domains of the D1 polypeptide, only Asp-59, Asp-61, Glu-65, His-92, Asp-170, Glu-189, and His-190 exert any significant influence on oxygen evolution under the conditions of our experiments. Of these residues, we have identified only Asp-170 as a likely ligand of the Mn cluster and His-190 as a possible ligand. However, because D170V, D170L, and D170I cells evolve oxygen at significant rates, we cannot exclude the possibility that Asp-170 does *not* ligate the *assembled* Mn cluster, although we do not favor this possibility. Therefore, at least 11 of the 14 coordination positions on the Mn cluster that are not occupied by μ_2-oxo bridging ligands in current models (Yachandra et al., 1993; DeRose et al., 1994) are occupied by a combination of carbonyl oxygens, water molecules, amino acid residues from the D1 polypeptide's carboxy-terminal domain, residues from polypeptides other than D1, and possibly residues other than Asp, Glu, His, and Tyr in the D1 polypeptide's interhelical domains.

The residues Asp-59, Asp-61, Glu-65, and His-92 influence the catalytic efficiency of the Mn cluster without significantly affecting its stability or its ability to assemble. The residue Glu-189 exerts some influence on the stability and/or assembly of the Mn cluster. However, this residue is unlikely to ligate either Mn or Ca^{2+}. Nevertheless, this residue profoundly influences the catalytic properties of the Mn cluster, perhaps by participating in a crucial network of hydrogen bonds. Both Asp-59 and Asp-61 may ligate a Ca^{2+} ion. However, if Asp-170 is a ligand to the Mn cluster, the ligation of Ca^{2+} by Asp-59 and Asp-61 would imply either that Ca^{2+} is located far from the Mn cluster or that the PSII core contains more than one Ca^{2+} ion. The extreme light sensitivity of D59N and D61A cells propagated in the absence of Ca^{2+} ions compared to mutants that lack Mn (*e.g.*, D170A) suggests either that a Ca^{2+} ion binds tightly to PSII in the absence of the Mn cluster or that, when oxidized, perturbed Mn clusters in Ca^{2+}-poor D59N and D61A cells release toxic activated oxygen species such as hydrogen peroxide. The binding of Ca^{2+} is also influenced by His-92 and Glu-189 (and, to a much lesser extent, by Asp-170), but none of these residues is likely to ligate Ca^{2+}. Finally, His-190 profoundly influences the redox properties of Y_Z, in addition to its possible role as a Mn ligand or as a crucial base or hydrogen bond donor.

The results obtained in this study will guide future studies of isolated mutant PSII particles to residues of particular interest in terms of Mn or Ca^{2+} ligation (*e.g.*, Asp-59, Asp-61, Asp-170, His-190) or other properties (*e.g.*, Glu-65, Glu-189, His-190) and to mutants that contain the most stable or most efficiently assembled Mn clusters *in vivo*.

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