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Lumenal side histidine mutations in the D1 protein of Photosystem II affect donor side electron transfer in *Chlamydomonas reinhardtii*

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Abstract

Site-directed mutants of the D1 protein generated in *Chlamydomonas reinhardtii* have been characterized to determine whether specific lumenal side histidine residues participate in or directly influence electron transfer. Histidine 195 (H195), a conserved residue located near the amino-terminal end of the D1 transmembrane α-helix containing the putative P680 chlorophyll ligand H198, was changed to asparagine (H195N), aspartic acid (H195D), and tyrosine (H195Y). These H195 mutants displayed essentially wild-type rates of electron transfer from the water-oxidizing complex to 2,6-dichlorophenolindophenol. Flash-induced chlorophyll *a* (Chl *a*) fluorescence yield rise and decay measurements for Mn-depleted membranes of the H195Y and H195D mutants, however, revealed modified Y_Z to $P680^+$ electron transfer kinetics. The rate of the variable Chl *a* fluorescence rise was reduced approximately 10-fold in H195Y and H195D relative to the wild type. In addition, the rate of Chl *a* fluorescence decay in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea was approximately 50-fold more rapid in H195D than in the wild type. These results can be accommodated by a change in the midpoint potential of Y_Z^+/Y_Z which is apparent only upon the removal of the Mn cluster. In addition, we have generated a histidine to phenylalanine substitution at histidine 190 (H190), a conserved residue located near the lumenal thylakoid surface of D1 in close proximity to the secondary donor Y_Z. The H190F mutant is characterized by an inability to oxidize water associated with the loss of the Mn cluster and severely altered donor side kinetics. These and other results suggest that H190 may participate in redox reactions leading to the assembly of the Mn cluster.

Key words: Electron transfer; Photosynthesis; Photosystem II; Chlorophyll *a* fluorescence; Site-directed mutagenesis

1. Introduction

The membrane-spanning polypeptides D1, D2, cytochrome *b*-559 and two low-molecular-weight polypeptides constitute the core of the Photosystem II reaction center complex [1–4]. A D1/D2 heterodimer is thought to coordinate the primary electron donor P680, as well as secondary donors and acceptors that participate in photochemically induced electron transfer from water to plastoquinone (for reviews on PS II, see [5–7]). Excitation of P680 promotes the transfer of an electron to a nearby acceptor, pheophytin (Pheo) within a few picoseconds [8]. This transient charge separation ($P680^+/Pheo^-$) is rapidly (within 200 ps) stabilized by the reduction of bound primary plastoquinone Q_A, followed by the oxidation of the secondary donor Y_Z (10–1000 ns, see [5]). Y_Z, a tyrosine residue (Y161) on the D1 protein [9,10], serves as an electron transfer intermediate [11,12] between $P680^+$ and the charge accumulating Mn cluster of the water-oxidizing complex.

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Abbreviations: Chl, chlorophyll; DPC, diphenyl carbazide; DCPIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, the electrochemical midpoint potential; H195D, H195N, and H195Y, mutants of *Chlamydomonas reinhardtii* with aspartic acid, asparagine, and tyrosine substitutions at position 195 of the D1 protein of Photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; P680 and $P680^+$, the reduced and oxidized forms of the primary donor of Photosystem II; PS II, Photosystem II; Q_A, primary acceptor of Photosystem II; Q_B, secondary acceptor of Photosystem II; Y_D and Y_Z, redox active tyrosines 160 and 161, respectively, of the D1 and D2 proteins of PS II.

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Although the tyrosine Y_Z has been established as an essential component in the electron transfer pathway [12], the question remains as to whether other amino acids in the reaction center core participate directly in or influence electron flow near P680. We have mutagenized specific amino acids (histidines) of the D1 protein to determine whether these individual residues influence the electronic properties of the P680 trap or redox-active components on the donor side of PS II.

P680, which may be either a dimer [13], or a monomer [14], of Chl *a*, is surrounded by a protein matrix that determines its orientation and distance with respect to the other PS II components. Because the redox potential of the $P680/P680^+$ couple is highly positive ($E_m \geq +1.0$ eV), the protein environment surrounding P680 is assumed to determine, at least in part, properties of the bound chlorophyll molecule(s). Similarly, the low oxidation potential ($E_m = +0.480$ eV) of the primary donor (P), in the purple bacterial reaction center has been partly attributed to the effects of its protein environment. Site-directed mutations of one [15,16] or several [17] amino acids in the vicinity of bacterial P, also called the special pair, have resulted in altered spectral properties, electron transfer kinetics, and redox potentials of the P/P^+ couple. Although these amino acid substitutions have revealed no essential residue requirement for reaction center function, it is apparent that the local protein environment does affect the electronic properties of the special pair. In PS II, amino acid residues that could account for significant shifts in electron density distribution on the chromophore or alter the redox potential of electron donors and acceptors include those which might hydrogen bond or have electrostatic interactions with these components. We have focused our efforts on histidine residues located in the vicinity of P680 on the lumenal side of the D1 protein. Histidine residues which are likely candidates to alter electron transfer on the oxidizing side of PS II include two conserved residues, H190 and H195 [18].

Residue H195 of the D1 protein is located at the amino terminus of the α-helical transmembrane span D and is potentially charged. Based on analogies to the bacterial reaction center model [1,2,19] this transmembrane span contains one of the histidine residues (H198) which is predicted to be a ligand to P680. The imidazole ring of H195 is therefore likely to point in the direction of P680. Another conserved histidine residue, H190 of the D1 protein, is located in the lumen-exposed polypeptide loop connecting helix C and helix D. A histidine residue (H190(D2)) is also conserved in the analogous position in the D2 protein. Several functions have been proposed for H190 based on its position on D1 including Mn ligand, chlorophyll ligand, hydrogen bonding to Y_Z [20], and as an oxidized intermediate in functional water oxidation [21]. Here we report the photosynthetic phenotypes of two histidine 195 (D1) mutants of *C. reinhardtii*, H195N (his-asn) and H195D (his-asp), as well as the previously described H195Y (his-tyr) mutant [18]. In addition, we describe the photosynthetic phenotype of the histidine 190 (D1 protein) mutant, H190F (his-phe).

2. Methods

C. reinhardtii strain *CC*-125 (wild type) was provided by the *Chlamydomonas* Genetics Center (Duke University). The wild-type strain that greens in the dark, CC-2137, was provided by R. Spreitzer (University of Nebraska). Plasmid p228, containing the chloroplast 16S rRNA gene conferring spectinomycin resistance [22], was provided by E. Harris (Duke University).

Plasmids containing the point mutations (pH190F, pH195N and pH195D) were constructed by oligonucleotide-directed mutagenesis of a 3.0 kb *Xba*I fragment of the *psbA* gene in a pBS^+ (Bluescribe) vector as previously described [18]. Cotransformation of *C. reinhardtii* chloroplasts with p228 and the plasmids containing mutagenized *psbA* fragments was performed via particle bombardment as described earlier [18], with the exception that a helium driven particle inflow gun [23] was used to deliver DNA-coated tungsten particles into the cells. For the H195D and H190F mutants, the wild-type strain CC-2137 was utilized as recipient in order to facilitate the selection of transformants in complete darkness. The H195N mutant was generated in the strain CC-125 as described for H195Y [18].

Each *psbA* point mutation generates a new restriction endonuclease recognition site in the chloroplast genome. H195Y, H195N and H195D contain unique *Rsa*I, *Xmn*I, and *Ava*II sites, respectively, created by basepair redundancy at a single position in the DNA sequence. The mutagenic oligonucleotide primers for H195N and H195D are: CCTTATG*AAC*CCATTCCA and CCTTATG*GAC*CCATTCCA. H190F contains a unique *Eco*RI site due to the CAC to TTC substitution in the oligonucleotide TTCCAAGCAGAA *TTC*AACATCC. Primary identification of spectinomycin resistant cotransformants was carried out by restriction site analysis of PCR-generated *psbA* fragments. Whole cell DNA lysates were prepared as previously described [18]. PCR products were then generated with 22 bp synthetic oligonucleotide primers corresponding to sequences within exon 4 located 52 bases 5′(GGTACTTTCAACTTCATGATCG) and 146 bases 3′(GGTTACCGTTTCGGTCTAGAAG) from the mutagenesis site (Fig. 1). The resulting 196 bp fragments were subsequently digested with the appropriate restriction endonuclease and electrophoresed in 2% agarose gel in TBE. The identification of single colony

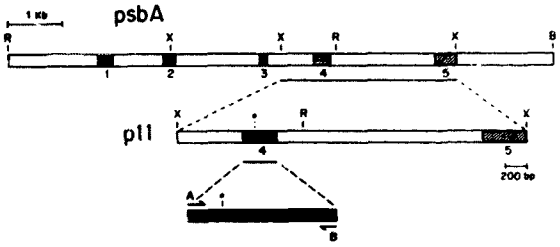


Fig. 1. Mutagenesis and screening of the *Chlamydomonas* psbA gene encoding the D1 protein. A 3 kb *Xba*I restriction fragment containing exons 4 and 5 of the *psbA* gene (p11) was mutagenized by oligo-directed nucleotide substitution as described [18]. X, *Xba*I; R, *Eco*RI; B, *Bam*HI; (*), approximate location of unique restriction sites created by H195 or H190 mutations; A, B, oligonucleotide primers for PCR restriction site screening. Exons are indicated by shading.

clones as homoplasmic for the point mutation was confirmed by Southern blot analysis and DNA sequencing.

C. reinhardtii thylakoids were prepared essentially as in Roffey et al. [18]. Cell cultures were grown in low light (8–12 $\mu E m^{-2} s^{-1}$) or in complete darkness in Tris-Acetate-Phosphate media at 25°C so as to avoid possible photoinhibitory effects. Cells were harvested at approximately $5 \cdot 10^6$ cells/ml, washed once with buffer A (0.35 M sucrose/25 mM Hepes (pH 7.5)/1 mM $MgCl_2$), and then resuspended at 1 mg/ml chlorophyll in buffer A containing 20 μM RNase A. After one passage through a French press (3000 lb/in^2), the suspension was centrifuged for 30 s at $1200 \times g$ to remove cell debris. Thylakoid membrane fragments were then pelleted by centrifugation for 12 min at $12000 \times g$. The pellet was gently resuspended in buffer B (0.4 M sucrose, 25 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mg/ml BSA) at 1.0–2.0 mg/ml Chl. PS-II-enriched membranes (BBY-type) were prepared by Triton X-100 solubilization according to Shim et al. [24]. Prior to Mn quantification, PS-II-enriched membranes were washed twice at 0.2 mg/ml Chl in buffer B containing 0.4 M sucrose, 20 mM Mes (pH 6.0), 5 mM EDTA, pelleted at $40000 \times g$, and resuspended in the same buffer at 1 mg/ml Chl.

Western blots of thylakoid preparations were performed essentially according to Towbin et al. [25] at chlorophyll amounts ranging from 0.1–10 μg chlorophyll in order to obtain a linear response to the antibody. The antibody used was raised against a synthetic peptide corresponding to amino acids 333–342 of the D1 protein [3] and antigen antibody complexes were detected using alkaline phosphatase labeled goat anti rabbit sera and a Promega detection kit. The intensity of the color reaction was determined by densitometry.

Maximal rates of steady-state oxygen evolution were determined for thylakoid membranes as previously described in Ref. 18. Assay mixtures containing 5–10 μg Chl/ml were illuminated with saturating (1600 $\mu E m^{-2} s^{-1}$) white light.

Thylakoid preparations were depleted of functional Mn by NH_2OH extraction. Membranes were washed once in buffer C (25 mM Hepes (pH 7.6); 1 mM $MgCl_2$, 0.4 M sorbitol) containing 1 M NaCl, pelleted in a microfuge, and then incubated [illegible] containing 2 mM NH_2OH for 15 min in the dark. Extracted membranes were washed three times in buffer C to remove traces of NH_2OH and resuspended in buffer C to approximately 1.0 mg/ml Chl.

DCPIP photoreduction was measured spectrophotometrically for Mn-depleted thylakoids in the presence of 1.0 mM diphenyl carbazide and 10 μM gramicidin. Assays were performed at pH 7.5 (buffer C) using a Chl concentration of 5 μg/ml; DCPIP concentration was 25 μM.

The rise and decay of Chl *a* fluorescence on the microseconds time scale after single-turnover actinic illumination was measured on a laboratory version of the instrument described by Kramer et al. [26] which was constructed in house. Thylakoids or PS II membranes in buffer C were used for all fluorescence yield measurements at 5 μg Chl/ml.

3. Results

Although *C. reinhardtii* cells contain a single chloroplast, there are 50–80 copies of the 196 kb chloroplast genome per cell. The *psbA* gene is located in the inverted repeat region of the genome, and so can be present in as many as 160 copies/cell. Fig. 2 illustrates the procedure used to identify and subsequently isolate homoplasmic lines of the *psbA* point mutations. Potential chloroplast transformants were primarily identified on the basis of spectinomycin resistance encoded by the 16S rRNA gene cotransformed into *C. reinhardtii* chloroplasts. The H195D mutation was generated by a CAC-GAC transition which resulted in the creation of an additional *Ava*II restriction site (-GGACC-) in the *psbA* gene. Identification of site specific mutants was facilitated by restriction site analysis of PCR amplified *psbA* sequences, a procedure which was approximately 3-fold more rapid than Southern blot analysis. Digestion of a 197 bp PCR fragment, corresponding to amino acid positions 178–242, with *Ava*II generates two DNA fragments (146 and 52 bp) in mutagenized DNA. The *Ava*II digested PCR fragments from the DNA extracts of several spectinomycin resistant colonies exhibited the DNA fragments diagnostic of the H195D mutation (Fig. 2A). The low intensity of the diagnostic bands was presumably due to a larger proportion of the WT chloroplast genome than that of the mutant. Single colony clones of the initial isolate re-

tained copies of both the mutant and WT *psbA* gene through two rounds of clonal propagation, maintaining a heteroplasmic condition (Fig. 2B). Subsequent screening of single colony clones following the third round of segregation led to the identification of isolates that appeared to be homoplasmic for the point mutation (Fig. 2C). The presence of the new *Ava* II site in all copies of the *psbA* gene was confirmed by Southern blot analysis of purified chloroplast DNA (Fig. 2D). Similar results were obtained in screens for the H195N and H190F mutants. These mutations resulted in the introduction of *Xmn*I and *Eco*RI restriction endonuclease recognition sites, respectively (not shown). Physical characterization of the H195Y mutant was described previously [18]. All nucleotide substitutions have been verified by DNA sequencing of PCR-derived products of exon 4.

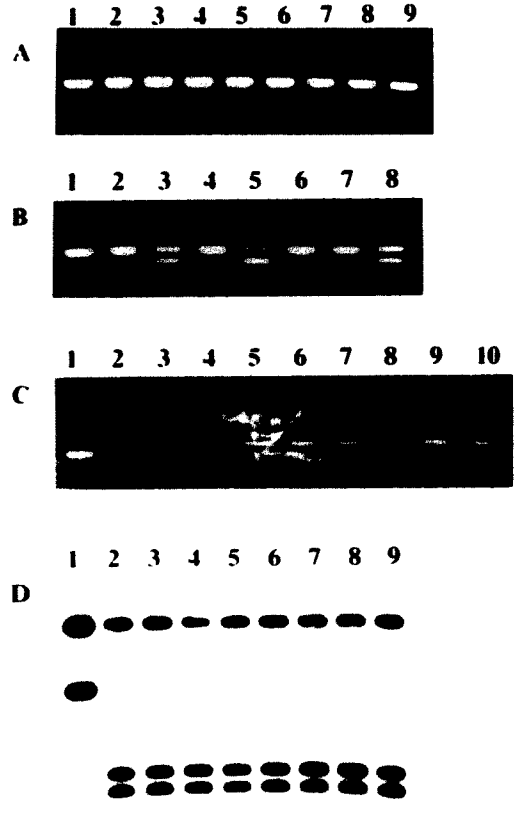


Fig. 2. Identification of a psbA-H195D mutant by PCR and Southern blot analysis. (A) *Ava*II digests of 197 bp PCR amplified DNA fragments from spectinomycin resistant *Chlamydomonas* transformants. The isolate in lane 7 was identified as a cotransformant heteroplasmic for the *psbA* mutation by the appearance of a 146 bp fragment diagnostic of the unique *Ava*II site created by a histidine to aspartic acid mutation at position 195. (B,C) Successive clonal derivatives of the original heteroplasmic cotransformant identified in A were screened for *psbA* heteroplasmy by PCR. In (B), the derivative in lane 5 (B,5) shows greater than 50% mutant *psbA* PCR fragments. In (C), the isolate in lane 8 (derivative of B,5) has nearly 100% mutant *psbA* PCR fragments and was further screened for *psbA* heteroplasmy by Southern blot analysis using a p11 probe. (D) Southern blot of *Ava*II digested total DNA from WT and H195D derivatives probed with plasmid p11. Lane 1, WT; lanes 2–9, H195D isolates (derivatives of C, 8).

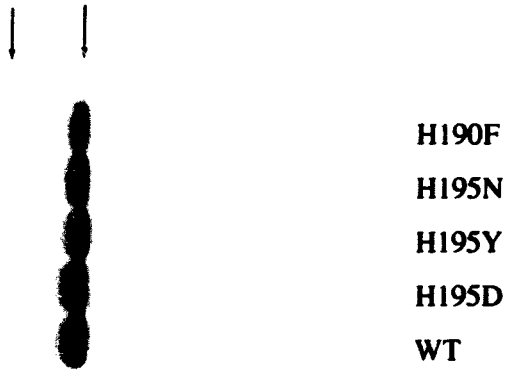


Fig. 3. Western blot of thylakoid proteins from the WT and the H195 and H190 mutants. Membrane proteins were separated by SDS-PAGE, electroblotted onto PVDF membranes, and probed with antibodies specific for the C-terminus of the spinach D1 protein [18]. The arrows indicate polypeptides cross-reacting with D1 antisera. The upper band corresponds to the 32 kDa D1 protein and the lower band to the 21 kDa D1 product of photoinhibition.

To determine whether the modification of the D1 protein had any effect on its steady-state level in thylakoid membranes, we quantified the amount of D1 protein in thylakoids by western blots. A representative western blot of one of several made using samples with different chlorophyll concentrations is shown in Fig. 3. As indicated in Table 1, there was no apparent difference in the amount of immunologically-detected D1 protein between the WT and the mutants. Therefore, it is unlikely that any differences in the electron trans-

Table 1
PSII characteristics of H195 mutants. Relative D1 protein content of WT and H195 mutants was determined by densitometry scans of western blots of membrane proteins from dark-grown cells. The results are the average of 3 separate determinations. Electron transport measurements were performed using isolated thylakoids as described in methods. O_2 evolution rates represent average rates for 5 different thylakoid preparations. Wild type rates of DPC → DCPIP electron transport, 43 μeq DCPIP.mg Chl^{-1}. h^{-1} (thylakoids)

	WT	H195N	H195Y	H195D
D1 protein (%WT)	100	102	94	110
O_2 evolution (%WT)	100	95	82	74
(μmol O_2.mg Chl^{-1}.h^{-1})	202 ± 17	192 ± 15	166 ± 31	149 ± 42
DCPIP reduction with DPC as a donor ($DCPIP_{red}$ (%WT))	100	105	91	95

port properties are the result of reduced numbers of PS II complexes per unit chlorophyll. There was, however, a low level of an additional, immunologically detectable band visible at approx. 22–25 kDa (Fig. 3), in the H190F mutant. This peptide corresponds in size to the 23 kDa fragment which is generated during donor side photoinhibition [27,28].

3.1. H195 mutants

Electron transport properties of H195 mutants

All H195 mutants were able to grow autotrophically on medium lacking acetate as a reduced carbon source. Growth rates on solid medium were similar to those of the wild type, revealing no significant impairment of photosynthetic capacity in these mutants. To determine the effects of the point mutations on PS II electron transfer in isolated thylakoid membranes, we measured maximal rates of oxygen evolution under saturating light conditions in the presence of uncouplers of photophosphorylation and artificial electron acceptors. Steady-state rates of oxygen evolution for the H195 mutants and the wild type are shown in Table 1. Rates measured for the H195Y and H195D mutants, grown under dark or low light conditions (8–12 $\mu E m^{-2} s^{-1}$), were 82 and 74% of the WT rates, respectively. Photosynthetic oxygen evolution rates were 60 and 40% of the WT for H195Y and H195D, respectively, when the cultures were grown at higher light intensities (> 40 $\mu E m^{-2} s^{-1}$) (data not shown). Oxygen evolution rates for the H195N mutant grown under [illegible] were similar to the rates measured for the wild-type strain. The apparent loss of oxygen-evolving capacity in light-grown H195Y and H195D cultures suggests that these mutants may be susceptible to photoinhibition. However, we found no increased sensitivity to strong light of the flash-induced Chl *a* fluorescence rise in the H195 mutants (data not shown).

To determine whether any modification in the donor side kinetics could be detected in the absence of the water-oxidation complex, electron transfer rates of DCPIP reduction using the artificial PS II donor DPC were determined in Mn-depleted thylakoids. The initial steady-state rates of DCPIP photoreduction for all

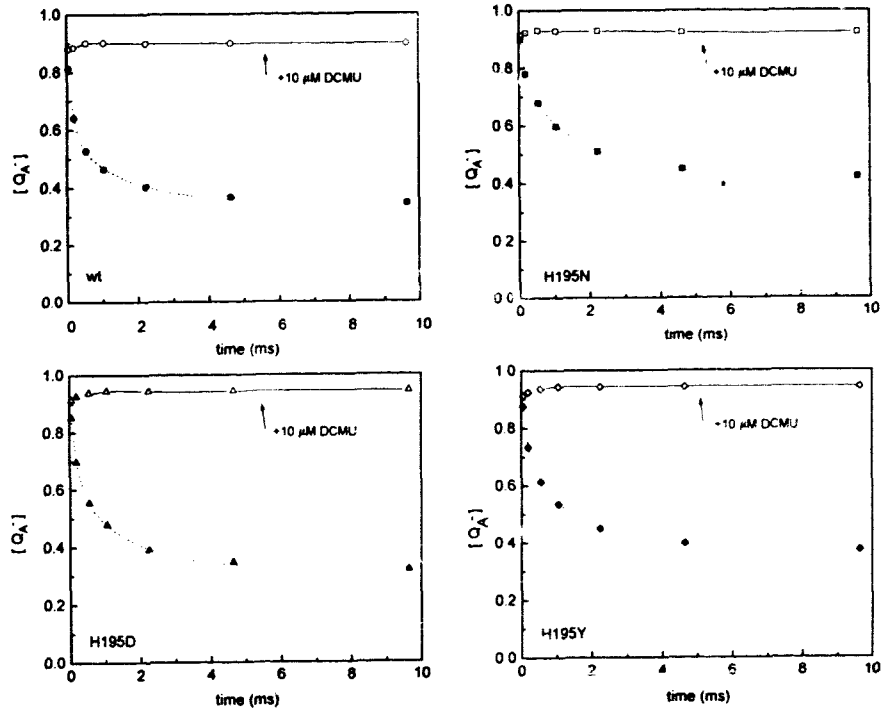


Fig. 4. Flash-induced fluorescence kinetics in oxygen-evolving thylakoids of WT and the H195 mutants. Saturating single-turnover flashes were given at time zero and the fluorescence yield changes associated with the formation and decay of the high fluorescence state, $P680Q_A^-$, were followed with a series of weak measuring pulses as described in Methods. Thylakoids were suspended at 5 μg/ml chlorophyll in buffer C (pH 7.5) and dark-adapted for 5 min prior to each experiment. The fluorescence yield changes were corrected for non-linearity as in [32] to approximate the concentration of the state $P680Q_A^-$. WT, wild type; H195D mutant; H195N mutant; H195Y mutant. In each figure, the closed and open symbols refer to experiments performed in the absence and presence, respectively, of 10 μM DCMU to block the oxidation of Q_A^- by Q_B.

of the mutants, measured within 10 seconds of illumination, were not significantly different from those of the wild type (Table 1). These results suggest that these modifications of H195 do not significantly alter the steady-state charge transfer capability of PS II.

Flash-induced chlorophyll a fluorescence kinetics of H195 mutants

To determine whether there were subtle effects on the donor side kinetics in the H195 mutants, we measured Chl *a* fluorescence rise and decay after single-turnover flash excitation. By following the yield of Chl *a* fluorescence, we can monitor the formation and decay of the high fluorescence state $P680Q_A^-$ (reviewed in, for example, Ref. [26]). $P680^+Q_A^-$, formed immediately following excitation, is a low fluorescence state due to the presence of $P680^+$, a quencher of fluorescence [29]. The subsequent increase in Chl *a* fluorescence reflects the reduction of $P680^+$, which results in the formation of the high fluorescence state, $P680Q_A^-$ [29]. The kinetics of the fluorescence rise are complex. A rapid phase (20–1000 ns, depending on the S-state of the center) has been attributed to the equilibrium of $P680^+/P680$ with the Y_Z^+/Y_Z couple (see for example, Refs. [26,30]). The slower phases (30 μs–1 ms) appear to follow the rate of S-state turn-over and have been attributed to the reduction of a small fraction of $P680^+$ in equilibrium with Y_Z^+ (reviewed in Refs. [26,31]). The amplitude of the slower phases then, is thought to reflect the equilibrium constant between Y_Z and P680, while its kinetics are thought to reflect the turnover of the S-states. In the absence of inhibitors, the rate of Chl *a* fluorescence decay following the formation of $P680Q_A^-$ depends on the rate of electron transfer from Q_A^- to the secondary acceptor Q_B. In the presence of DCMU the decay presumably reflects recombination of Q_A^- with S states.

Fig. 4 shows the kinetics of flash induced fluorescence changes in dark-adapted thylakoids of the wild type and the H195 mutants. As is seen for the wild type, the rapid phase of the Chl *a* fluorescence rise in the mutants was complete before the first time point at 40 μs, suggesting that electron transfer from the S states of water-oxidizing complex (S_n) to Q_A^- is not severely affected, in agreement with the oxygen evolution and artificial donor (DPC) assays. The fluorescence decay curves shown in Fig. 4 can be described by two exponentials and a residual (longer than 2 s) which may arise from non-Q_B reducing centers [31]. The decay constants and amplitudes for these phases (Table 2) were similar for the mutants and the wild type, revealing no significant effect on the acceptor side ($Q_A^-Q_B$ to $Q_AQ_B^-$) of PS II.

In the presence of the herbicide DCMU, oxidation of Q_A^- by Q_B is blocked, and the rate of fluorescence decay depends upon the rate of charge recombination between Q_A^- and oxidized donor side components. No significant differences were seen in the slow phase of the fluorescence rise kinetics between the WT and the H195 mutants (Fig. 4). If our interpretation of these kinetics is correct (see Refs. [26,30,31] and above), these data imply that no substantial changes in the equilibrium between Y_Z and P680 or the kinetics of the $S_1 \rightarrow S_2$ turnover (after the first flash) were induced by the H195 mutations. In Fig. 5, the fluorescence decay measured after a series of single-turnover actinic flashes in the presence of DCMU is shown on a longer time scale. It reveals a slightly more rapid decay of the high fluorescence state for H195D and H195Y than for the wild-type and H195N thylakoids.

In the absence of the Mn cluster, formation of $Y_Z^+P680Q_A^-$ following a single-turnover flash occurs within 10 ms at pH 7–8 [33,34]. The fluorescence rise kinetics measured with non-oxygen-evolving thylakoid preparations in the presence of DCMU reflect the

Table 2
Fluorescence decay constants and amplitudes for WT and H195 mutants measured for oxygen-evolving thylakoids. Values in parentheses are amplitudes

	Phase 1	Phase 2	Phase 3
WT	145 μs (0.35)	1530 μs (0.27)	> 2 s (0.38)
H195N	157 μs (0.20)	1628 μs (0.35)	> 2 s (0.43)
H195Y	170 μs (0.34)	1537 μs (0.34)	> 2 s (0.40)
H195D	196 μs (0.31)	1590 μs (0.31)	> 2 s (0.36)

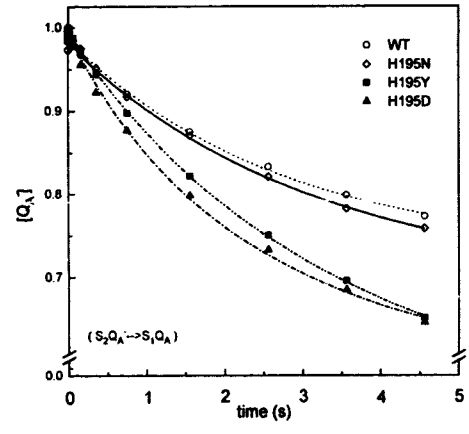


Fig. 5. Kinetics of recombination of $S_2Q_A^-$ to S_1Q_A measured by the decay of flash-induced fluorescence in WT and H195 mutant thylakoids. Thylakoids were dark-adapted for 5 min prior to addition of 10 μM DCMU, and then further dark-adapted for 5 min. After dark adaptation, the majority of PS II centers are expected to be in the state S_1Q_A; after flash excitation, the state $S_2Q_A^-$ is expected to be formed in the majority of enters (see text). The decay of this state was measured by following the fluorescence decay after single-turnover actinic flash, and correcting for non-linearity as in [32]. (open circles), wild type; (closed squares), H195Y mutant; (closed triangles), H195D mutant; (open diamonds), H195N mutant.

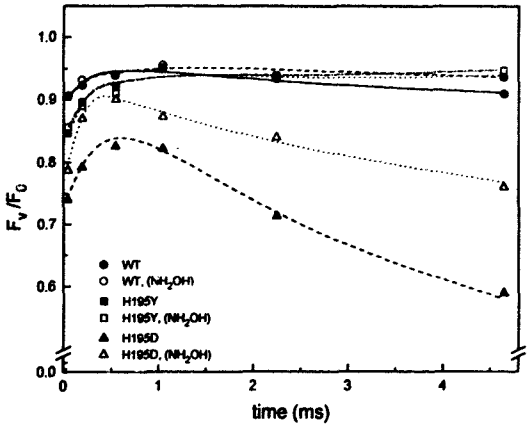


Fig. 6. The effect of hydroxylamine addition on the flash-induced fluorescence rise in Mn-depleted thylakoids from the WT and H195 mutants. Conditions were as in Fig. 4, in the presence of 10 μM DCMU, but in the absence and presence of 5 mM NH_2OH added as an exogenous PS II donor (see text) to dark-adapted thylakoids. The closed and open symbols refer to experiments performed in the absence and presence of NH_2OH; (circles), wild type; (squares), H195Y mutant; (triangles), H195D mutant. The fluorescence yield was normalized to F_{max} after 2nd flash.

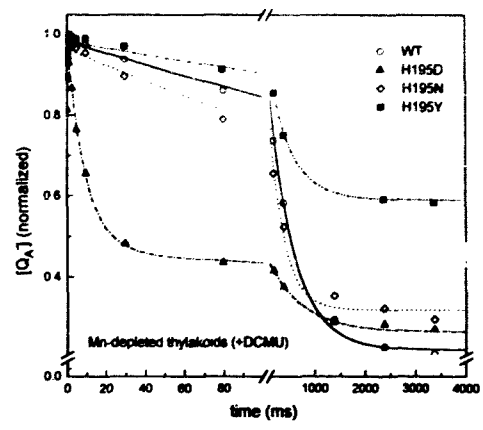


Fig. 7. Kinetics of recombination of $Y_Z^+Q_A^-$ to Y_ZQ_A measured by the decay of flash-induced fluorescence in Mn-depleted thylakoids of the WT and the H195 mutants. NH_2OH-extracted thylakoids (see Methods) were assayed as in Fig. 4 in the presence of 10 μM DCMU. After dark adaptation for 5 min, the Mn-depleted PS II centers are expected to be in the state Y_ZQ_A; after flash excitation, the state $Y_Z^+Q_A^-$ is expected to be formed in the majority of centers (see text). The decay of this state was measured by following the fluorescence decay after single-turnover actinic flash, and correcting for non-linearity as in [32]; (open circles), wild type; (closed squares) H195Y mutant; (closed triangles), H195D mutant; (open diamonds), H195N mutant.

$Y_ZP680^+ \rightarrow Y_Z^+P_{680}$ electron transfer rate. In Mn-depleted thylakoid preparations of the H195D and H195Y mutants, the rise of variable Chl *a* fluorescence was sufficiently slowed so as to be detectable on a microsecond time scale (Fig. 6). We could not, however, accurately determine the half-times of the fluorescence rise due to instrument limitations. The apparent rates could be estimated, however, and were approximately 30–50 μs for the H195D and H195Y mutants. The fluorescence rise for H195N was similar to that of the wild type and was essentially complete before the first measuring point at 40 μs (not shown).

The decay of Chl *a* fluorescence in the non-oxygen-evolving membrane preparations was also measured to determine the extent to which the mutations altered the kinetics of $Y_Z^+Q_A^-$ recombination. Fluorescence decay curves for the NH_2OH-treated thylakoids are shown in Fig. 7. In the presence of DCMU, the decay of the high fluorescence state $P680Q_A^-$ for the wild type, and the mutants H195N and H195Y can be adequately fit by one exponential and a residual (Table 3). Only the more rapid exponential decay component ($t_{1/2} \approx 200$ ms) was resolved under the experimental conditions described. The relative amplitude of the slow residual phase(s) was significantly increased in the H195Y mutant. This slow decay could presumably represent the stabilization of the high fluorescence state by an alternate donor or donors. Based on this assumption, an increase in the contribution of the slow phase of fluorescence decay in the H195Y mutant could imply either a higher concentration of the donor or a more rapid rate of electron donation by the donor.

In the case of H195D, two exponential and one residual component were required to fit the data for the fluorescence decay (Fig. 7). The most rapid phase of the H195D decay kinetic ($t_{1/2} = 9.5$ ms) was approximately 50-fold more rapid than the decay for wild-type, H195N or H195Y thylakoids. If we assume that the intrinsic rate of $P680^+Q_A^-$ recombination was not affected by the mutation, we may argue that the rapid decay observed for the H195D mutant is due to a perturbation of the equilibrium between Y_ZP680^+ and Y_Z^+P680. The addition of a sufficient concentration of exogenous donor would be expected to shift the equilibrium toward states in which P680 is reduced (via Y_Z, see Discussion). We tested this prediction by observing the fluorescence rise after a single actinic flash in the absence and in the presence of an exogenous donor,

Table 3
Fluorescence decay constants and amplitudes for DCMU-treated Mn-depleted thylakoids from the WT and the H195 mutants. Values in parentheses are amplitudes

	Phase 1	Phase 2	Phase 3
WT	501 ms (0.76)	> 10 s (0.24)	
H195N	297 ms (0.65)	> 10 s (0.35)	
H195Y	452 ms (0.39)	> 10 s (0.61)	
H195D	9.5 ms (0.51)	668 ms (0.20)	> 10 s (0.26)

hydroxylamine (5.0 mM) (Fig. 6). We found that the fluorescence level for the wild type and H195Y, in the tens to hundreds of microseconds range was unaffected by the addition of NH_2OH. In the H195D mutant, on the other hand, the variable Chl *a* fluorescence level was approximately 5% higher in the presence of NH_2OH. The fluorescence yield is not a linear measure of the fraction of centers with reduced Q_A unless a correction is made for the substantial energy transfer between PS II centers [32]. Because we do not have a reliable measurement of the probability (p) that an exciton in antennae associated with a closed center will migrate to another center, we must treat the fluorescence level as a rough estimate of the concentration of oxidized P680 centers remaining after the flash. Based on these estimates, the increased fluorescence level in the H195D mutant upon the addition of an exogenous donor is consistent with a mutation-induced shift in the Y_ZP680^+-Y_Z^+P680 equilibrium constant which favors accumulation of Y_ZP680^+ relative to wild type.

3.2. H190F mutant

Table 4 is a summary of photosynthetic characteristics of the H190F, D1 mutant. The substitution of a histidine residue at position 190 with phenylalanine (H190F) resulted in the complete elimination of oxygen-evolving capability in whole cells and isolated thylakoids. The loss of oxygen evolution was not associated with a loss of PS II centers, since the D1 protein content was 90% of wild-type levels (Fig. 3). Furthermore, Coomassie-blue-stained gels of PS II particles indicated that the other PS II polypeptides were present at levels equivalent to wild type (data not shown).

Table 4
PSII characteristics of the WT and H190F mutant. Relative D1 content was measured by densitometry of western blots of thylakoids from dark grown cells. Electron transport measurements were performed using thylakoids (oxygen evolution) or PSII membranes (DCPIP reduction) as described in methods. The fluorescence yield following single turnover actinic flashes was measured as in [26]

	WT	H190F
Oxygen evolution (μmol O_2.mg Chl^{-1}.h^{-1})	220	–
D1 content/Chl (%WT)	100	90
Mn/200 Chl [a]	4.8	1.1
DCPIP reduction with DPC as a donor ($DCPIP_{red}$ (μEq.mg Chl^{-1}h^{-1}))	92	48 (45) [b]
S-state period 4 oscillations (fluorescence rise and decay)	+	–

[a] Elemental analysis of EDTA-washed PSII membranes by Inductively Coupled Plasma Spectroscopy was performed by REAL Labs, The Ohio State University, Wooster, OH.
[b] Rates of DPC oxidation for the H190F mutant were measured using NH_2OH-extracted and untreated PSII particles (in parentheses).

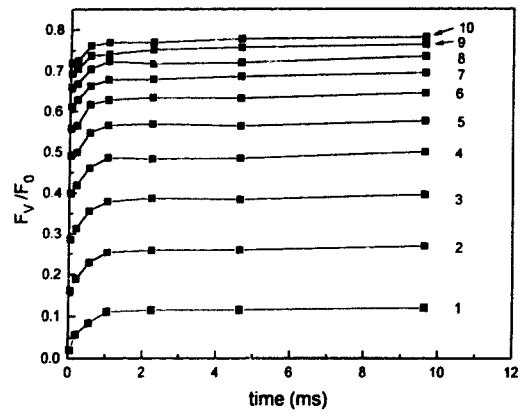


Fig. 8. Flash-induced fluorescence kinetics in the H190F mutant. Thylakoids were assayed as in Fig. 4 with 10 μM DCMU added to block oxidation of Q_A^- by Q_B, except that no attempt was made to correct the fluorescence rise for non-linearity. The fluorescence yield is instead expressed as F_v/F_0 (the variable fluorescence divided by the F_0 level). The accumulated Chl *a* fluorescence yield was measured after each of a series of 10 saturating single-turnover flashes given at 300 ms intervals. The number corresponding to each actinic flash in the series is indicated to the right of the traces.

In order to determine whether the mutation results in the disruption of electron transfer between Y_Z to Q_A, DCPIP photoreduction was measured in the presence of DPC as a donor. Both untreated and NH_2OH-extracted H190F thylakoids were able to oxidize DPC; however, the rate of DPC to DCPIP electron transfer was 50% less than that of the wild type. The presence of significant capacity to oxidize artificial donors to PS II in the presence of DCPIP indicates that electron transfer from Y_Z to Q_A is not disrupted. Furthermore, since identical rates were observed for untreated and NH_2OH-extracted thylakoids, Mn, which has been shown to inhibit photooxidation of DPC [35], is apparently absent or present in only very low concentrations in H190F PS II complexes.

Chlorophyll a fluorescence in the H190 mutant

The apparent loss of functional Mn as a result of the H190F mutation could reflect the loss of a Mn ligand or an inability to photooxidize Mn during the photoactivation process [36]. A comparison of H190F and wild-type $Y_Z \rightarrow Q_A$ electron transfer properties was made to define further the primary effect of the histidine to phenylalanine substitution at this position. Analysis of the Chl fluorescence rise and decay properties of the H190F mutant reveals a significant modification of the donor side kinetics. The variable Chl *a* fluorescence yield was extremely low (about 10% of that in the WT) for H190F thylakoid membranes following a single saturating flash in the presence of DCMU (Fig. 8). Furthermore, the Chl *a* fluorescence

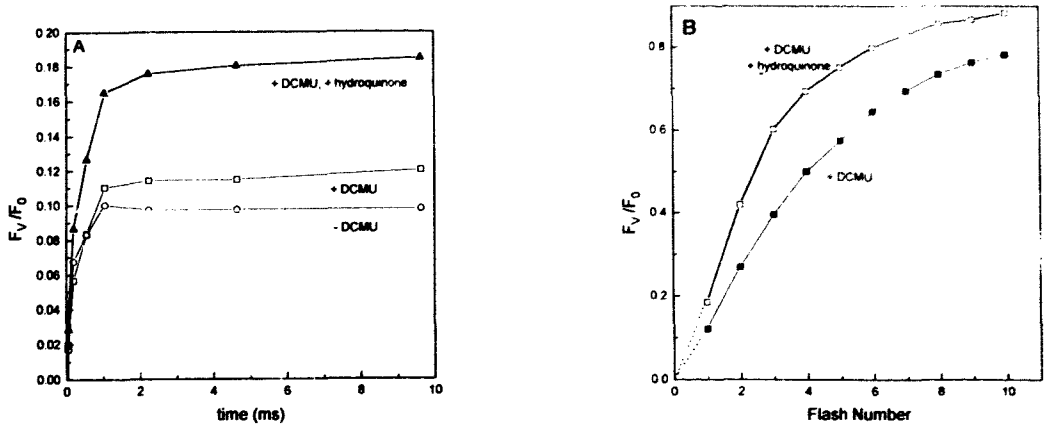


Fig. 9. The effect of an exogenous PS II donor on the flash-induced fluorescence changes in the H190F mutant. A. The effect on fluorescence rise kinetics. The assay conditions were as in Fig. 8 except for the addition of hydroquinone as an artificial donor to Y_Z^+. The Chl *a* fluorescence yield was measured in the absence (open circles) and presence (open squares) of 10 μM DCMU; and in the presence of 10 μM DCMU and 5 mM hydroquinone (closed triangles). B. The effect of flash number on the accumulation of the high fluorescence state. The assay conditions were essentially as in A. The fluorescence yield was measured in the presence of 10 μM DCMU (closed squares), and in the presence of 10 μM DCMU and 5 mM hydroquinone (open squares) 9–10 ms after each of a series of actinic flashes. The increase in fluorescence is plotted against the flash number to give an approximation of the efficiency of formation of $P680Q_A^-$.

rise ($t_{1/2} \approx 250$ μs) was approximately 10^4-fold slower than what would be expected for the wild type, based on an 30 ns half-time [30] for the rereduction of $P680^+$ in oxygen-evolving thylakoids, and 100-fold slower than in non-oxygen-evolving (NH_2OH-extracted) WT thylakoids (Fig. 8). Since, after the first flash, the fluorescence yield (F_V/F_0) curve rises from zero, we assume that there were no kinetically unresolved phases. Increasing the number of saturating actinic flashes in the presence of DCMU resulted in elevated levels of Chl *a* fluorescence (Fig. 8). These results indicate that $P680 \cdot Q_A^-$ can be accumulated, but at reduced quantum yield in the H190F mutant (see Discussion).

The addition of hydroquinone increased the Chl *a* fluorescence yield after a single flash but did not significantly alter the kinetics of the Chl *a* fluorescence rise (Fig. 9A). Upon further single flashes, a more rapid accumulation of the high fluorescence state could be observed (Fig. 9B).

The decay of the high fluorescence state in the presence of DCMU appears to be much longer ($t > 10$ s, Fig. 10) in the H190F mutant than in the wild type. Furthermore, as shown in Fig. 10, the decay kinetics measured after the 10th flash in the absence of DCMU are only slightly more rapid than those observed in the presence of DCMU. Since the fluorescence decay without DCMU should reflect $Q_A^-Q_B \rightarrow Q_AQ_B^-$ electron transfer, these results are an indication that the PS II acceptor side reactions have also been affected in the H190F mutant. Similar effects on acceptor side reactions have been observed in hydroxylamine extracted PS II particles and PS II particles depleted of the extrinsic proteins of the oxygen-evolving complex [7].

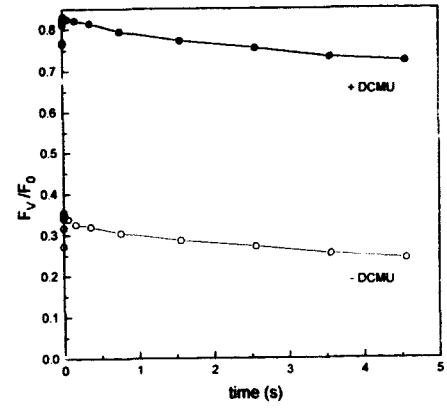
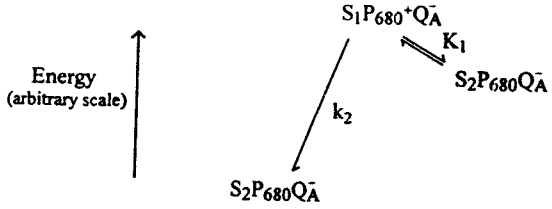


Fig. 10. Decay kinetics of the high fluorescence state in the H190F mutant. Thylakoids were treated as in Fig. 9A in the (closed circles) presence and (open circles) absence of DCMU, without the addition of an exogenous donor. After a series of 10 actinic flashes at 300 ms intervals, the decay of the high fluorescence state was monitored in the milliseconds to seconds time range by a series of weak measuring pulses.

4. Discussion

We have demonstrated that mutagenesis of specific lumenal side histidine residues of the D1 protein, H195 and H190, has resulted in modified electron transfer properties in PS II reaction centers in *C. reinhardtii*. Aspartic acid (H195D) and tyrosine (H195Y) substitu-



Scheme I. Model of the donor side electron transfer reactions in oxygen-evolving preparations of the H195 mutants. In this scheme, k_2 represents the rate of recombination of $P680^+Q_A^-$ and K_1 represents the equilibrium constant for reduction of $P680^+$ by the S-state complex. If K_1, which equals k_1/k_{-1}, is high in thylakoid preparations in which the Mn complex is intact, then the high fluorescence state $P680Q_A^-$ will be formed in nearly all PS II centers immediately after the flash. This was observed as a rapid fluorescence rise for the WT and the H195 mutants in the presence of DCMU (Fig. 4). The rate of decay of the high fluorescence state depends on the rate of recombination between Q_A^- and the S-states.

tions at H195 resulted in PS II centers that are capable of oxidizing water but display somewhat slower electron transfer to $P680^+$ as measured by Chl *a* fluorescence rise and decay in Mn-depleted thylakoids (see Figs. 6 and 7). However, an asparagine (H195N) substitution had little apparent affect on the donor side reactions. Since H195N and H195Y as well as the H195D mutants have nearly wild type levels of oxygen evolution, it would appear that under optimal conditions H195 is not required for normal electron transfer from the Mn cluster. Nevertheless, flash-induced Chl *a* fluorescence rise and decay kinetics indicate that H195 substitutions can effect electron donation to $P680^+$.

Based on the kinetics of the flash-induced Chl *a* fluorescence yield in oxygen-evolving thylakoids (plus DCMU), it is possible to determine the effects of the mutations on the rate of recombination from $S_2Q_A^-$ to S_1Q_A.

As defined in Scheme I, the ratio of the rate constants k_1/k_{-1} defines the equilibrium constant (K_1) for electron transfer from the S-states to $P680^+$. Since changes in K_1 or k_2 (the intrinsic rate constant for $P680^+Q_A^-$ recombination) should affect the rate of the back reaction from Q_A^- and therefore the kinetics of the decay of the high fluorescence state, we conclude, based on the data presented in Fig. 4, that only relatively small changes in either of these constants were induced by the H195Y and H195D mutations. A reduction in K_1 (not observed in the H195 mutants) could indicate that the midpoint potentials of the $P680^+$/P680 or S_2/S_1 couples was altered. Since the value of K_1 in the H195 mutants was essentially the same as in the wild type, it is unlikely that there was any effect on the midpoint potentials of $P680^+$/P680 or the S-state complex (see Fig. 5).

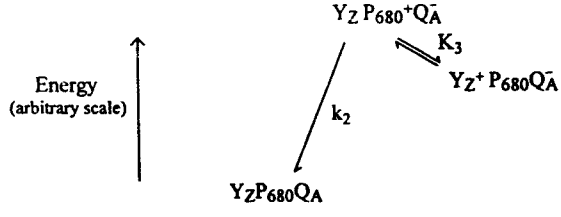
The most significant effect of the H195 mutations was on electron transfer in Mn-depleted thylakoid membranes. After flash excitation of the Mn-depleted membranes in the presence of DCMU, the state $Y_Z^+P680Q_A^-$ is formed in most centers. The half-time of formation of $P680Q_A^-$ observed in the H195D and H195Y mutants was estimated to be 30–50 μs (see Fig. 6). Since electron transfer from the water-oxidizing complex to Y_Z would be rate-limiting in oxygen-evolving preparations (half-times of 30 μs to 1300 μs [37] for S-state transitions), it is reasonable to assume that moderate mutation-induced changes in electron transfer from water to P680 may not be apparent except in Mn-depleted thylakoids.

We have used the following scheme II as a model for the recombination reactions in Mn-depleted PS II in the presence of DCMU as previously suggested [38,39].

The rate of recombination of $Y_Z^+P680 \cdot Q_A^-$ will depend on the rate of $P680^+Q_A^-$ recombination (k_2) and the concentration of $P680^+Q_A^-$, which is determined by the equilibrium constant K_3 for electron transfer between Y_Z and $P680^+$. When the rates of recombination were probed by following the decay of Chl *a* fluorescence, we found that the rates for wild-type, H195Y, and H195N (Fig. 7) were comparable to those previously measured in Tris-washed thylakoids [34,39]; however, the fluorescence decay in the H195D mutant was nearly 50-fold more rapid. Assuming the reaction between Y_Z and $P680^+$ reaches equilibrium on the time scale of the back reaction, we used the analysis of Yerkes et al. [39] to estimate the mutation-induced changes in either k_2 or K_3.

$$\nu = [Y_Z P680^+ Q_A^-] \cdot k_2 \quad (1)$$

where ν represents the experimentally determined rate estimated from the half-time of Chl *a* fluorescence decay. The equilibrium constant K_3 can be written in



Scheme II. Model of the donor side electron transfer reactions in Mn-depleted thylakoids of the H195 mutants. Here, K_3 is the equilibrium constant for the $Y_ZP680^+Q_A^- \Leftrightarrow Y_Z^+P680 \cdot Q_A^-$ reaction; see Scheme I and text for details. The model predicts that the addition of hydroxylamine as a donor to Y_Z^+ should shift the equilibrium, resulting in an increase in the yield of the high fluorescence state, as was observed experimentally (Fig. 6).

terms of the concentrations of the charge-separated states:

$$K_3 = \frac{[Y_Z^+ P680 Q_A^-]}{[Y_Z P680^+ Q_A^-]} \quad (2)$$

Since all reactions in the scheme are first order, at any time during the kinetics, we can normalize the total number of PS II centers which have not recombined to 1, so that the fraction of centers with oxidized Y_Z will be:

$$[Y_Z^+ P680 \cdot Q_A^-] = 1 - [Y_Z P680^+ Q_A^-] \quad (3)$$

Therefore, K_3 can be expressed in terms of the fraction of PS II centers with $P680^+$ remaining after a flash:

$$K_3 = \frac{1 - [Y_Z P680^+ Q_A^-]}{[Y_Z P680^+ Q_A^-]} \quad (4)$$

Combining Eq. 1 and Eq. 4, it is possible to estimate K_3 on the basis of k_2 and the experimentally determined rate of $Y_Z^+ P680 Q_A^-$ decay:

$$\nu = \frac{k_2}{K_3 + 1} \quad (5)$$

Based on the above treatment, it is apparent that either an increase in k_2 or a decrease in K_3 would result in an increase in ν. We assume, for the present, that the rate of recombination of $P680^+ Q_A^-$ (k_2) is not significantly affected by the alterations on the donor side. In this case, any change in ν reflects only changes in K_3. If we assume that $k_2 = 7000\ s^{-1}$ (based on $t_{1/2} = 130\ \mu s$, [39,40]), we obtain the values for K_3 shown in Table 5. For wild-type, H195N and H195Y thylakoids, K_3 was determined to be within a factor of 2 of that calculated in [39,40] for spinach thylakoids. The altered decay kinetics observed for NH_2OH-washed H195D membranes indicate an approximately 50-fold shift in the equilibrium (K_3) between Y_Z^+/Y_Z and $P680^+/P680$. Similar effects have been observed for K_3 in Mn-depleted WT membranes and attributed to an apparent change in the midpoint potential of the Y_Z^+/Y_Z couple [33,39–41].

The low K_3 value calculated for H195D is in agreement with the results in Fig. 6, which demonstrate the effects of the addition of an exogenous PS II donor on the Chl *a* fluorescence yield following flash excitation. In these experiments the difference between the fluorescence yield with and without added donor should reflect the fraction of centers in the $P680^+$ state following a flash. While both H195Y and the wild type showed no significant difference in the fluorescence yield, indicating a high equilibrium constant between $Y_Z P680^+$ and $Y_Z^+ P680$ (i.e., essentially [illegible] $P680^+$), the addition of the donor to the H195D thylakoids effectively shifted the equilibrium toward the states with reduced P680 (as indicated by a higher fluorescence yield). This result is also consistent with the assumption that k_2 is not altered by the modification of the donor side reactions.

The apparent shift in K_3 in H195D may be attributed to an effect on the redox potential of either $P680^+$ or Y_Z. The introduction of a negatively charged residue in the vicinity of P680 and Y_Z could conceivably act to stabilize the oxidized form of P680, thus altering the midpoint potential (E_m) of the $P680^+/P680$ couple. In thermoluminescence studies of the H195 mutants, however, we have found that the peak position of the A_T band, which is attributed to recombination between Q_A^- and an oxidized donor side component in NH_2OH-washed or Tris-washed thylakoids (for review, see Ref. [42]), was nearly identical in wild-type and all H195 mutants in this study (D.K., R.R., G., and R.S. (1994) Biochim. Biophys. Acta 1185, 228–237). Since the energetics of the recombination between Q_A^- and the donor side component associated with the formation of the A_T band would presumably be affected by any mutation-induced shift in the E_m of $P680^+/P680$, we argue against this type of an effect. Secondly, a change in the E_m of $P680^+/P680$ would be expected to result in a change in the rate of recombination from $S_2Q_A^-$ as well. As shown in Fig. 5, the kinetics of recombination between $S_2Q_A^-$ and S_1Q_A, although slightly modified in oxygen-evolving H195D and H195Y thylakoids, are less than 2-fold more rapid than that of WT and H195N. These results were in contrast to those obtained with Mn-depleted H195D thylakoids, which had a 50-fold slower rate of $P680^+$ reduction than WT. In summary, although we cannot completely exclude other possibilities, the model which best explains the data on the H195D mutant is that in Mn-depleted preparations, the midpoint potential of Y_Z, and not that of P680, is shifted from that of WT. Data from the fluorescence rise in the 30 μs range (Fig. 4) and $S_2Q_A^-$ recombination rates (Fig. 5) suggest that in intact PS II centers, the potential difference between $Y_Z^+ P680$ and $Y_Z P680^+$ was similar in H195D and WT.

Chlorophyll *a* fluorescence induction and decay kinetics were also significantly altered in the H190F mutant. Our analysis of the Chl *a* fluorescence kinetics

Table 5
Equilibrium constants for WT and H195 mutants. Values for ν, the rate of $Y_Z^+ P680 Q_A^-$ decay, are based on the phase 1 Chl *a* fluorescence decay constants (Table 3) for NH_2OH-extracted thylakoids. K_3 was calculated for $k_2 = 7000 s^{-1}$ ($\tau = 130\ \mu s$) based on Eq. 5

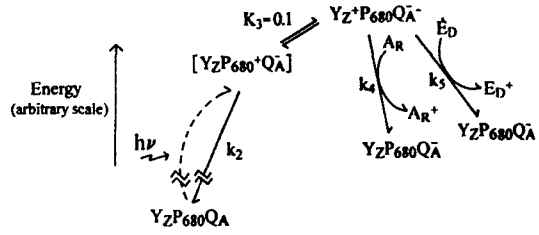
	ν	K_3
WT	2.0	3758
H195N	3.4	2205
H195Y	2.2	3409
H195D	105.3	72

in H190F is outlined in Scheme III and described below.

In the absence of the S-state complex, $P680^+$, a quencher of Chl *a* fluorescence [29] accumulates and we observe a low fluorescence yield immediately following an actinic flash. Two processes compete for the rereduction of $P680^+$: $Y_Z P680^+ Q_A^-$ recombination to $Y_Z P680Q_A$ (rate constant k_2) and the oxidation of Y_Z. Y_Z is then slowly reduced by an unidentified alternate donor, A_R (rate constant k_4). We propose that A_R is probably distinct from the alternate donor, A_D which gives rise to the A_T thermoluminescence band (note, the A_T band is not formed in the H190F mutant). A_R must be sufficiently reducing to allow the $Y_Z P680Q_A^-$ state to be present for a very long time, since the back reaction in the presence of DCMU was on the order of 10 s (Fig. 10). If the rate of donation by A_R is 5–10-fold slower than $P680^+ Q_A^-$ recombination, the fluorescence rise kinetics will be dominated by the intrinsic $P680^+ Q_A^-$ recombination rate ($k_2 \approx 7000 s^{-1}$ (based on $t_{1/2} = 130$ μs [34,38]). The fluorescence rise kinetics observed for the H190F mutant ($t_{1/2} \approx 250$ μs) are in agreement with this model, since we expect $P680^+ Q_A^-$ recombination to proceed with $t_{1/2} \approx 130$ μs.

In the presence of an efficient exogenous donor (E_D), the extent of $P680^+$ reduction after a flash (and therefore the high fluorescence state, $Y_Z P680Q_A^-$) would be expected to increase in the context of the above scheme. This prediction is borne out by the increase in efficiency for the formation of $Y_Z P680Q_A^-$ in the presence of hydroquinone (see Fig. 9B). The sum of the rate constants for the secondary donors A_R and E_D (k_4 and k_5, Scheme III) was estimated to be about 1.5-times that of k_4 alone, indicating that the competition between the $P680^+$ rereduction processes can be influenced through the reduction of Y_Z. Since the kinetics of fluorescence decay were similar in the presence and absence of DCMU it is apparent that Q_A^- to Q_B electron transfer was inhibited. Alterations in acceptor side kinetics by donor side modifications have also been documented in hydroxylamine extracted thylakoids as well as in thylakoids missing the extrinsic polypeptides of the oxygen-evolving complex. It is possible that Mn coordination places constraints on the possible orientations of transmembrane spans which in turn may affect acceptor side processes.

As has been shown here (Table 4) as well as in site-directed *Synechocystis* mutants [7,43] H190 (D1) is required for oxygen evolution. The Mn content of H190F PS II particles from dark-grown and briefly illuminated cells was determined to be 1–1.8 Mn/200 Chl, less than that required for oxygen evolution (Table 4). Since the D1 content of H190F mutant thylakoids was identical to that of the WT (Fig. 3, Table 4), the loss of a functional water-splitting apparatus is likely to be due to the loss of the Mn S-state complex and not



Scheme III. Model of the donor side electron transfer reactions in the H190F mutant. To account for the flash-induced fluorescence kinetics we have introduced a scheme where the low fluorescence state $Y_Z P680^+ Q_A^-$ (in brackets), formed immediately after the flash, can decay by several routes. k_2 represents the rate of decay by recombination of $P680^+$ with the electron in Q_A^-. K_3 represents the equilibrium constant for reduction of $P680^+$ by Y_Z. To account for the lack of rapid rise in fluorescence after the flash, we had set K_3 so that little of the high fluorescence state, $Y_Z^+ P680Q_A^-$ is formed (the equilibrium favors the state with oxidized P680). A high fluorescence state is accumulated by the reduction of Y_Z^+ by an alternate donor, A_R, present in the thylakoid preparation, with a rate constant k_4. The low quantum yield of formation of the high fluorescence state is accounted for in the model by making the reduction of Y_Z^+ slow so that recombination is considerably more rapid. For example, if we make $K_3 = 0.1$, then if $k_4 = 3000$, only 10% of centers will end up in the high fluorescence state $Y_Z^+ P680Q_A^-$ after a saturating single-turnover actinic flash, similar to what was observed (see Fig. 8). Additional flashes are predicted to result in the accumulation of high fluorescence states as observed (Fig. 8). The model predicts that the addition of an exogenous donor, E_D, should allow a more efficient accumulation of the high fluorescence state since the reduction of Y_Z^+ would be expected to compete more effectively with recombination. This is also observed experimentally (see Fig. 9B).

to the loss of PS II centers (Table 4). This interpretation is supported by the observation of De Vitry et al. [44] using PS II reaction center mutants, that the D1 protein turns over rapidly in unassembled PS II complexes. Finally, period-four oscillations associated with the S-state cycling in the oxygen-evolving complex which were observed for WT cells were not observed for the H190F mutant (data not shown).

The loss of a functional Mn S-state complex in H190 mutants indicates that H190 (D1) may be required for coordination or photoligation of Mn of the S-state complex. Based on distance estimates determined from magnetic coupling between Mn-Y_Z,Y_D [45], chemical modification [46], mutagenesis [47] and cross-linking experiments [48] it has been suggested that the D1 protein provides at least some of the ligands for binding the Mn complex (see also Ref. [49]). EXAFS spectroscopy of oxygen-evolving PS II particles from spinach indicates that Mn ions are coordinated by oxygen or nitrogen atoms at distances between 1.8 and 2.2 Å [50]. Carboxylate groups capable of stabilizing the higher Mn oxidation states are likely to contribute the bulk of Mn ligands, but imidazole groups of histidine residues have also been proposed as ligands [49,51]. Recently, a structural model for the Mn cluster has been proposed

by Yachandra et al. [52] in which a histidine residue is assigned as a terminal Mn ligand based on electron spin echo envelope modulation studies on EPR signals from cyanobacterial PS II particles grown in $^{14}NO_3$ and $^{15}NO_3$ [53]. Site-directed mutagenesis of conserved amino acids in the lumenal regions of D1 and D2 in cyanobacteria also suggest that histidine residues may be required for Mn binding and/or photoactivation of the Mn cluster [51,54,55]. Histidines on the D1 protein, which have been shown to be required for functional water oxidation include H332, H337, and H190 [7,43]. However, unlike all other lumenal side D1 histidine mutants with impaired oxygen evolution no amino acid replacements have been identified for H190 which partially restore oxygen evolution (Richard Debus, personal communication). These results suggest that the structure of the putative Mn-H190 complex may preclude substitution by other amino acid residues. Other lines of evidence, however, argue against Mn coordination by H190. The distance between Y_Z and the Mn cluster is estimated to be ≥ 10–15 Å [56] based on EPR measurements. This distance, however, is larger than the 4–5 Å distance between H190 and Y_Z predicted by recent models for the donor side of PS II [20]. Since the donor side models are based on homologies within the transmembrane regions of D1/D2 and the L/M subunits of the bacterial reaction center, the orientation of residues outside these regions is difficult to predict. Therefore, the possibility that H190 is a Mn ligand on the D1 protein cannot be excluded on this basis.

The loss of the Mn S-state complex may also be attributed to the loss of a redox active residue required for donor side electron transfer and possibly Mn photoligation. Padhye et al. have proposed that a histidine residue mediates electron transfer between the Mn-cluster and Y_Z [57]. However, we have observed formation of Y_Z^+ in H190F and H190Y mutants (R.R., K.J. van Wijk, R.S. and S. Styring, unpublished data). These results suggest that H190 does not participate in donor side reactions between Y_Z and P680. Other lines of evidence also suggest that histidine residues may participate in donor side reactions. The appearance of a new EPR signal in Ca^{2+}-depleted PS II suggests that a histidine residue may be oxidized during the S_2 to S_3 transition [21]. In addition, the A_T thermoluminescence band observed at -20°C in Mn-depleted PS II has been attributed to charge recombination between Q_A^- and an oxidized histidine residue [58,59]. In recent thermoluminescence studies using *Chlamydomonas* thylakoids and PS II particles, we have observed a loss of the A_T band in the H190F mutant (D.K., R.R., G., and R.S. (1994) Biochim. Biophys. Acta 1185, 228–237). These results suggest that H190 may be redox active in thylakoids with a non-functional S-state complex. However, due to the drastically altered $P680^+$ rereduction kinetics in H190F we cannot rule out the possibility that the loss of the A_T band is due to substantially modified electron transfer reactions.

In summary, the H190 mutant: (1) does not evolve oxygen; (2) has a low Mn content in the PS II particles; (3) has substantially altered Y_Z to $P680^+$ electron transfer rates; (4) is missing the A_T thermoluminescence band; and (5) has increased sensitivity to photoinhibition associated with donor side inhibition of electron transfer [59–64]. These results may be attributed to a requirement for H190 as a redox active residue in the assembly of the water-oxidizing Mn cluster.

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