Aspartate 170 of the Photosystem II Reaction Center Polypeptide D1 Is Involved in the Assembly of the Oxygen-Evolving Manganese Cluster†

Peter J. Nixon‡ and Bruce A. Diner*

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

Received October 3, 1991; Revised Manuscript Received October 30, 1991

ABSTRACT: Eleven site-directed mutations were constructed at aspartate 170 of the D1 polypeptide of the photosystem II (PSII) reaction center of the cyanobacterium *Synechocystis* sp. PCC 6803. The light-saturated rates of O_2 evolution (V_{O_2}) measured in whole cells range from close to that of wild-type for Asp170Glu to zero for Asp170Ser and Ala. Those mutant strains that are best able to evolve O_2 are also those that show the lowest K_m in PSII core complexes for the oxidation of Mn^{2+} by oxidized Tyr161, the normal oxidant of the Mn cluster responsible for O_2 evolution. To a first approximation, the lower the pK_a of the residue at position 170, the higher the V_{O_2} and the lower the K_m. D1-Asp170 appears to participate in the early steps associated with the assembly of the Mn cluster. It is also the first reported example of an amino acid residue critical to the function and assembly of the oxygen-evolving complex.

The reaction center of photosystem II (PSII)[1] is one of the two natural photovoltaic cells that drive electron transfer in the electron transport chain of oxygenic photosynthesis [for a recent review, see Hansson and Wydrzynski (1990)]. Photoexcitation of the primary electron donor, P_{680} (a chlorophyll *a* or pair of chlorophylls *a*), results in an electron transfer to the primary electron acceptor, a pheophytin *a*. The electron is then transferred to the primary plastoquinone electron acceptor, Q_A, and subsequently to the secondary plastoquinone electron acceptor, Q_B. These reactions bear a strong kinetic and spatial resemblence to those that occur on homologous components in the reaction centers of the purple non-sulfur photosynthetic bacteria (Michel & Deisenhofer, 1989; Komiya et al., 1988). The crystallographic structure of reaction centers from two such sources has been recently solved to atomic resolution (Michel & Deisenhofer, 1989; Yeates et al., 1988; Allen et al., 1988; El-Kabbani et al., 1991). The electron donor side of the PSII reaction center is, however, quite different from that of the photosynthetic bacteria. Much higher oxidizing potentials ($E_m \sim 1.2$ V) are generated by the oxidized form of the primary electron donor, P_{680}, to allow the oxidation of a redox active tyrosine, Y_z, which in turn oxidizes a cluster of four manganese ions responsible for the oxidation of water to molecular oxygen [for a recent review, see Debus (1991)]. It is the identification of a site implicated in the early ligation and assembly of the Mn cluster that form the subject of this paper.

In accordance with the electrochemistry of water oxidation, the reaction center must undergo four one-electron photoreactions to produce the four oxidizing equivalents required for the generation of an oxygen molecule (Kok et al., 1970; Joliot & Kok, 1975]. The oxidation state of the oxygen-evolving complex (OEC) is designated by S_n states where the subscript (0–4) refers to the number of oxidizing equivalents that have accumulated (Kok et al., 1970). The dark-stable state is S_1. Release of an oxygen molecule accompanies the one dark transition from S_4 to S_0, resetting the counter to zero. At least two of the oxidizing equivalents are temporarily stored in the form of oxidized Mn with the remaining two possibly stored in the form of organic free radicals (Debus, 1991).

Structural information on the Mn cluster obtained using EXAFS indicates Mn–Mn interatomic distances of ~2.7 and 3.3 Å (Sauer et al., 1988; George et al., 1989; Penner-Hahn et al., 1990), consistent, respectively, with di μ-oxo and mixed μ-oxo and carboxylato- bridges observed in synthetic complexes. The coordinating ligands are thought to be primarily oxygen with some nitrogen (DeRose et al., 1991) and possibly a chloride. At least half of the 20–24 total coordinating ligands are probably supplied by water and by amino acid coordination. The most likely candidates among the latter are Asp, Glu, His, and Tyr, all of which have been identified in other manganoproteins (Wieghardt, 1989).

Neither the amino acid residues nor the subunit(s) responsible for coordination of the Mn cluster have been identified. Indirect evidence points to a role for polypeptide D1, one of two polypeptides (D1 and D2) responsible for the coordination of the primary photoreactants of the PSII reaction center (Nanba & Satoh, 1987; Marder et al., 1987). Polypeptide D1 contains the immediate oxidant Y_z of the Mn cluster (Babcock et al., 1976; Gerken et al., 1988), identified as Tyr161 (Debus et al., 1988; Metz et al., 1989), which is reversibly oxidized to the neutral free radical species (Babcock et al., 1989) and is probably located within 10–15 Å of the Mn cluster (Hoganson & Babcock, 1988). It is also known that proteolytic processing of the carboxy terminus of the D1 polypeptide is required before the Mn cluster can be assembled

† This paper is contribution no. 6085 of the Central Research and Development Department of the E. I. du Pont de Nemours Co. We gratefully acknowledge the support of the USDA/CRGO.

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

* Address correspondence to this author.

[1] Abbreviations: Chl, chlorophyll *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, midpoint potential; EPR, electron paramagentic resonance; EXAFS, extended X-ray absorption fine structure; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; P_{680}, primary electron donor, a monomer or dimer of chlorophyll a; PCR, polymerase chain reaction; Pheo, pheophytin a, the primary electron acceptor; PSII, photosystem II; Q_A, primary plastoquinone electron acceptor; Q_B, secondary plastquinone electron acceptor; S_n, oxidation states of the oxygen-evolving complex where *n* represents the number of stored oxidizing equivalents; Y_z, secondary electron donor, D1-Tyr161.

0006-2960/92/0431-942$03.00/0

Table I: Comparison of D1-Asp170 Mutants

strain[a]	photosynthetic growth[b]	[RC] (% of WT per cell)[c]	light-saturated O_2 rate (% of WT per Chl)[d]	O_2/flash, oscillation of period 4[e]	S_2 lifetime in whole cells (s)[f]	K_m (μM; in core complex)[g]	pK_a of residue (in H_2O)[h]
WT (TC31)	+	100	100	yes	20 ± 5	1	4–5
D1-Asp170Glu	+	100	60–100	yes	65 ± 5	2	4–5
D1-Asp170His	+ (weak)	≥80	20–40	yes	45 ± 5	2–10	6
D1-Asp170Cys	–	≥70	20–30[i]	nd[j]		1	12
D1-Asp170Tyr	–	≥50	10–20	yes	55 ± 5	20–40	9–11
D1-Asp170Arg	–	≥60	10–20	yes	30 ± 5	5	12–13
D1-Asp170Met	–	≥70	10–20	yes	65 ± 10	20	
D1-Asp170Trp	–	5–10	3–5			nd	
D1-Asp170Asn	–	≥60	0–2			50	25
D1-Asp170Ala	–	≥70	0			50	
D1-Asp170Ser	–	≥70	0			60	18
D1-Asp170Phe	–	10	0			nd	

[a] See Materials and Methods for construction of strains. [b] Measured in BG-11 medium according to Williams (1988). WT cells have a doubling time of 20 h when grown under photoautotrophic conditions. [c] See Materials and Methods for the measurement conditions and an explanation of the protocol. [d] Measured in BG-11 medium plus 5 mM glucose containing 0.3 mM 2,6-dichlorobenzoquinone and 1 mM $K_3Fe(CN)_6$ as described under Materials and Methods. The wild-type rate of saturated steady-state oxygen evolution was typically 150–200 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}. [e] Yes indicates the normal advance of the S_n states of the OEC with O_2 released on every fourth flash. [f] Measured on a centrifugal Joliot-type rate electrode (Joliot & Joliot, 1968) according to Forbush et al. (1971) by giving a single flash followed by a variable time interval and detection of O_2 on the second member of a flash pair given 270 ms apart. [g] Measured as in Figure 4b. [h] From March, 1968. [i] Susceptible to light inactivation of O_2 evolution. [j] nd, not determined.

(Diner et al., 1988; Taylor et al., 1988). Finally, none of the site-directed mutants that have been constructed at Asp, Glu, Asn, and Gln residues on the lumenal side of polypeptide D2 completely eliminate O_2 evolution except where the reaction center is also lost (Pakrasi & Vermaas, 1991).

We, as well as Richard Debus (personal communication), have targeted for site-directed mutagenesis many of the potential Mn-coordinating residues within D1. Out of the 14 carboxylate-containing residues, and six histidines located on the side of polypeptide D1 facing the thylakoid lumen, we have now identified four residues at which site-directed amino acid substitutions specifically block water oxidation. In this paper, we report on our analyses of the site-directed mutations that have been made at one of these positions, D1-asp170. [Preliminary results on two of the mutants have been reported (Nixon & Diner, 1990)]. The other sites will be described in separate publications.

Materials and Methods

The glucose-tolerant strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988) was used for the construction of the site-directed mutants described in this paper. Site-directed alterations were constructed in the *psbA3* gene using the oligonucleotide-mediated in vitro mutagenesis protocol of Kunkel et al. (1987). The mutant gene was then restored through homologous recombination to the chromosome of a *psbA*-triple deletion strain of *Synechocystis* sp. PCC 6803 following transformation by standard methods (Williams, 1988). Further details of the strains and plasmids that were used will be described in a forthcoming paper (Nixon et al., in preparation). Mutants were confirmed by DNA gel blot analysis, PCR sequencing, and where possible by "complementation" tests in which small fragments of the wild-type *psbA3* gene were used to restore the mutant to photoautotrophy. All strains were grown in BG-11 media as described by Williams (1988) and Metz et al. (1989). Five millimolar glucose was included in the media to allow the propagation of mutants defective in PSII.

Optical spectroscopy was performed using a flash-detection spectrophotometer based on a design by Joliot et al. (1980) and described in Metz et al. (1988). Actinic flashes were provided by a Candela SLL250 dye laser using either Rhodamine 6G or Oxazine 720 as the lasing dye.

Measurements of the relative quantum yield of chlorophyll fluorescence were performed using the same flash-detection spectrophotometer described by Metz et al. (1988). Weak probe flashes (422 nm) were used to follow the kinetics of relaxation of the fluorescence yield following saturating actinic light flashes. The same technique was also used to determine the relative PSII reaction center concentration per cell (Table I) which is proportional to the total variable fluorescence yield at a fixed cell concentration. Cells (0.9 OD at 730 nm) were preincubated in BG-11, 5 mM glucose, 50 mM HEPES-NaOH (pH 7.5), *p*-benzoquinone (0.3 mM), and $K_3Fe(CN)_6$ (0.3 mM). After a 10-min incubation, 40 μM DCMU was added, followed 1 min later by 20 mM NH_2OH. Illumination by 15 saturating flashes (18 Hz) occurred 30 s after addition of 20 mM NH_2OH. Hydroxylamine assured the reduction of the oxidizing equivalents on the donor side, blocking charge recombination with Q_A^-. The number of flashes and the high flash frequency were chosen to assure complete reduction of Q_A by the end of the flash series, the point at which the fluorescence yield is measured. The variable fluorescence is the difference in the fluorescence yield measured after minus that measured before illumination.

Oxygen flash yields were measured on whole *Synechcocystis* cells using a Joliot-type rate electrode (Joliot & Joliot, 1968) specially designed to allow centrifugation of the cells onto the electrode surface. The cells were suspended in BG-11 plus 5 mM glucose containing 100 mM KCl and loaded onto the electrode, which was then spun for 5 min in a swinging bucket rotor (Sorvall HS-4) at 4000 rpm. The cells were allowed to dark adapt for 5 min and were then excited with unfiltered saturating xenon flashes (Stroboslave 1539A, Gen Rad).

Steady-state rates of oxygen evolution were measured in saturating light on whole *Synechocystis* cells using a thermostated Hansatech oxygen electrode. Cells were grown to a cell density corresponding to 2–3 OD at 730 nm as described in Nixon et al. (1991). The cells were then assayed in BG-11 medium (at 2–10 μg of chl/mL) containing 5 mM glucose plus 0.3 mM 2,6-dichloro-*p*-benzoquinone and 1 mM $K_3Fe(CN)_6$. The concentration of chlorophyll *a* present in the sample was determined spectrally by extracting the cell pigments with methanol and using an extinction coefficient of 79 mM^{-1} cm^{-1} at 665 nm (Lichtenthaler, 1987). The zero level of light-saturated steady-state oxygen evolution was determined using a *psbA* triple deletion strain of *Synechocystis* 6803 (no PSII

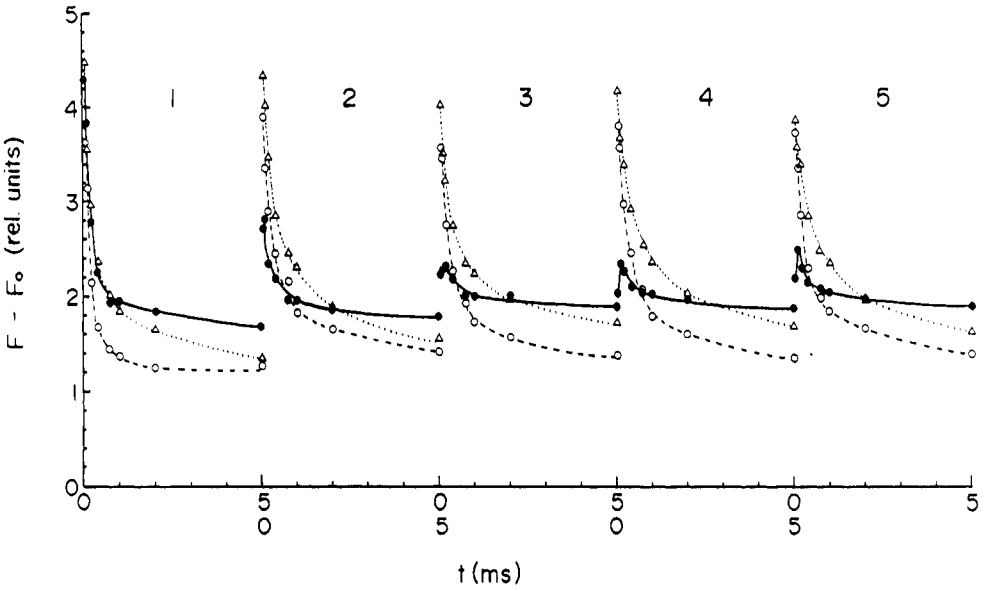

FIGURE 1: Relaxation of the variable fluorescence ($F - F_0$) after each of a series (1.67 Hz) of five saturating 2-μs flashes in whole *Synechocystis* cells. Shown is the 5-ms range following each flash starting at 50 μs. WT (○) is compared to mutants D1-Asp170Glu (△) and D1-Asp170Ser (●), the most and least active with respect to O_2 evolution. Mutants showing intermediate levels of inhibition of O_2 evolution show intermediate levels of quenching at 50 μs on the second and subsequent flashes. Whole cells were suspended at 0.9 OD at 730 nm in growth medium (BG-11 plus 5 mM glucose). A 10-min dark incubation with 0.3 mM *p*-benzoquinone plus 0.3 mM $K_3Fe(CN)_6$ preceded the start of measurement. The 50-μs values of the variable fluorescence normalized to F_0 $[(F - F_0)/F_0]$ following the first flash are 0.33, 0.35 and 0.25 for the WT, D1-Asp170Glu, and D1-Asp170Ser strains, respectively.

reaction centers). Measurements using this mutant gave no transient upon turning the actinic light on or off. The lower limit of detection of oxygen evolution was about 5 $\mu M \cdot h^{-1}$.

Photosystem II core complexes were isolated according to the procedure of Rögner et al. (1990).

RESULTS

Mutations at D1-Asp170. The *psbA3* gene of *Synechocystis* 6803 was engineered as described under Materials and Methods so that Asp170 of polypeptide D1 was replaced by 11 different residues. Only two of the substitutions were able to grow photoautotrophically:Asp170Glu and Asp170His. Asp170Glu grew at near wild-type rates, while Asp170His grew poorly with a doubling time approximately three times greater than the 20 h observed for wild-type (WT).

Measurements in whole cells of oxygen evolution coupled to the electron acceptors 2,6-dichloro-*p*-benzoquinone and ferricyanide showed, however, that some of those strains incapable of photosynthetic growth were still able to evolve oxygen though at depressed rates. The ranking of the relative light-saturated rates of oxygen-evolution are indicated in Table I. Most of the mutants, with the exceptions of Asp170Phe and Asp170Trp (5–10% of WT levels), showed levels of PSII reaction centers comparable to that of WT. A ranking of Asp170Trp based on O_2 evolution rates per center would consequently increase to 30–100% of WT. Mutants D1-Asp170Ser, Ala, and Phe show no O_2 evolution. The Ser and Ala replacements are, therefore, the first site-directed PSII mutants to be constructed in the reaction center that retain the reaction center but entirely block water oxidation.

Those amino acid replacements that retain some oxygen activity are also those which are the most likely to ligate metals. The guanidinium group of Arg would be an unusual ligand. However, Arg has recently been shown to be capable of ligating Pt(II) (Ratilla & Kostic, 1988) and tetramethylguanidine binding to Cu(II), Co(II), Zn(II), Pd(II), Ni(II), and Cr(II) has also been reported (Longhi & Drago, 1965). The trend in O_2-evolving activity appears to approximately follow that of the pK_a of the residue at D1-170, where the higher activity is shown by those residues with the lower pK_a.

Kinetics of Relaxation of the Fluorescence Yield of Chlorophyll. We have used the time-dependent variation of the quantum yield of chlorophyll fluorescence following saturating flash excitation to further characterize the site of lesion in whole cells of these mutants. The fluorescence yield is a sensitive measure of the oxidation state of redox components associated with the PSII reaction center. The state $P_{680}PheoQ_A$ gives a low quantum yield of fluorescence (F_0, typically 3% in green plants and algae) and that of $P_{680}PheoQ_A^-$, the highest fluorescence yield (F_{max}, typically 10%). The dependence of the fluorescence yield on $[Q_A^-]$ is nonlinear where energy transfer occurs between centers but linear in the absence of energy transfer (Joliot & Joliot, 1964). The latter situation appears to prevail in *Synechocystis* 6803. With Q_A reduced, an equilibrium is rapidly established following excitation, with excitation energy distributed between A* (excited chlorophyll in the antenna), P_{680}^* (the lowest singlet excited state of the primary electron donor) and $P_{680}^+Pheo^-$ (the primary charge separated state) (van Gorkom, 1985). The energy is dissipated in part by fluorescence primarily from A* on the nanosecond time scale.

The charge-separated state $Y_zP_{680}^+PheoQ_A^-Q_B$, generated within 300 ps of excitation of P_{680}, is initially in a low fluorescence state (F_0) despite reduction of Q_A. This is because P_{680}^+ quenches the fluorescence (Butler et al., 1973). The fluorescence yield then increases to the maximum level [F_{max}, $t_{1/2}$ ~30 ns (Mauzerall, 1972)] with the reduction of P_{680}^+ by donor Y_z to form $Y_z^+P_{680}PheoQ_A^-Q_B$ (Gerken et al., 1988; van Best & Mathis, 1978). Reduction of Y_z^+ by S_1 has little effect on the fluorescence yield; however, oxidation of Q_A^- by electron transfer to Q_B [$t_{1/2}$ = 100–200 μs (Robinson & Crofts, 1983)] lowers the fluorescence yield again (approaching F_0) with the formation of the state $Y_zP_{680}PheoQ_AQ_B^-$.

The existence of these different fluorescence states provides a means for detecting and characterizing mutants that are impaired in the OEC. In the absence of the Mn cluster, the first light-driven charge separation produces the state $Y_Z^+P_{680}PheoQ_A^-Q_B$ within 40 μs (Reinman et al., 1981), with

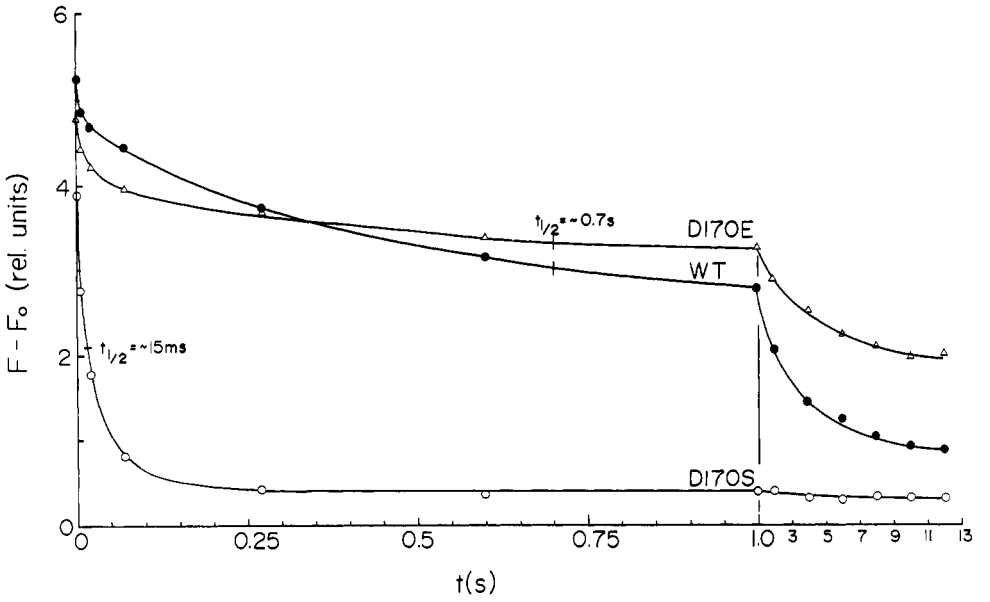

FIGURE 2: Relaxation of the variable fluorescence ($F - F_0$) resulting from charge recombination between Q_A^- and the PSII donor side following a single saturating 2-μs flash excitation of whole cells of WT (●), D1-Asp170Glu (△), and D1-Asp170Ser (○) in the presence of 40 μM DCMU. Mutants showing intermediate levels of inhibition of O_2 evolution show fast phases of charge recombination intermediate in amplitude between that of D1-Asp170Ser and WT. The cells were treated as in Figure 1, except that DCMU was added at least 1 min before the start of measurement. The variable fluorescence normalized to F_0 $[(F - F_0)/F_0)]$ at 200 μs, the earliest time point, is 0.49, 0.38, and 0.26 for the WT, D1-Asp170Glu, and D1-Asp170Ser strains, respectively.

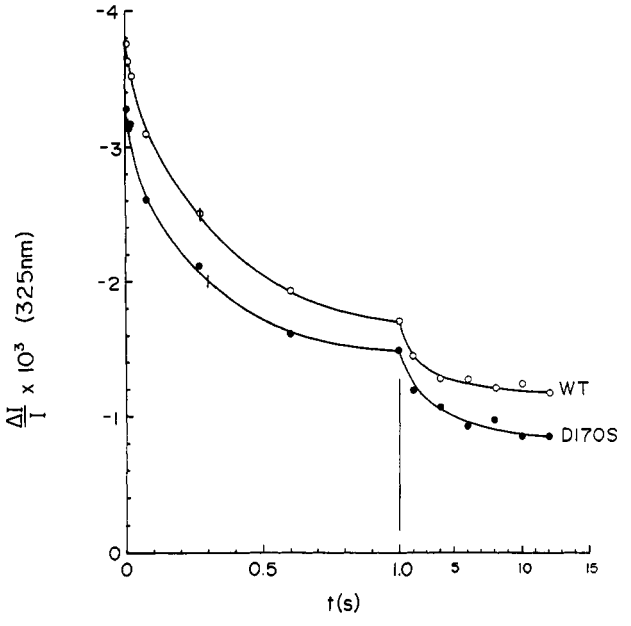

FIGURE 3: Kinetics of charge recombination between Q_A^- and the PSII donor side following a single 2-μs flash (≥85% of saturation) excitation of PSII core complexes of WT (○) and D1-Asp170Ser (●) at pH 7.5. Measurements were made at the absorbance maximum (325 nm) of the difference spectrum of Q_A^-–Q_A. Core complexes (10 μM chlorophyll) were suspended in 20 mM HEPES-NaOH, pH 7.5, containing 1 μM $K_3Fe(CN)_6$.

Y_z^+ unable to be reduced by a tertiary electron donor. A second photoexcitation given before Y_z^+ can be rereduced by charge recombination with Q_B^- will produce $Y_z^+P_{680}^+$-$PheoQ_A^-Q_B^-$, which will remain close to the F_0 level, despite the reduction of Q_A. By looking for the appearance of a low fluorescence state in a series of light flashes at 50 μs after the actinic flash, it is possible to count the number of available electron donors to P_{680}. Fifty microseconds is long relative to the reduction time of P_{680}^+ when the OEC is intact [$t_{1/2} \approx 50$ and 260 ns (Brettel et al., 1984)] but short relative to the rate of oxidation of Q_A^- by Q_B [$t_{1/2}$ = 100–200 μs (Robinson & Crofts, 1983)] and to recombination between P_{680}^+ and Q_A^- [$t_{1/2}$ = 1 ms (Metz et al., 1989)]. The fluorescence yield will remain high at 50 μs after each flash of a series until $Y_z^+P_{680}^+$ is formed.

Absence of a Teriary Electron Donor. An example of such an assay is shown in Figure 1 for the D1-Asp170Ser, Asp170Glu, and WT strains. The fluorescence yield is shown for each of a series of five flashes (1.67 Hz) and is measured between 50 μs and 5 ms after each flash. The 0.6-s spacing between actinic flashes was chosen as it is long enough for completion of slow phases in Q_A^- oxidation by Q_B but short enough to prevent loss of long-lived Y_z^+ in the absence of the OEC. Both the WT and Asp170Glu strains show a near constant fluorescence yield detected at 50 μs after each actinic flash. The Asp170Ser mutant, however, shows a quenching of fluorescence at 50 μs that appears on the second and subsequent flashes, indicating that following the first flash there is an appreciable fraction of centers that have not reduced Y_z^+ before the arrival of the second flash. This observation imples that in many centers there is no tertiary electron donor functioning in the Asp170Ser mutant. All of the mutants show behavior that ranges between that of Asp170Ser and that of Asp170Glu, with those that are the most impaired with respect to oxygen evolution showing the greatest extent of quenching in the flash series.

Another indicator of the limitation of electron donation to the PSII reaction center is the rate of charge recombination between a light flash-generated oxidized electron donor and Q_A^- in the presence of an inhibitor [e.g., DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]] that blocks oxidation of Q_A^- by Q_B. Following a single flash, the dark-stable S_1 state of the OEC is oxidized to S_2 on the electron donor side of WT reaction centers and Q_A^- is generated on the electron acceptor side. Charge recombination between Q_A^- and the S_2 state of the OEC occurs with a $t_{1/2}$ of ~1 s in WT cells (Figure 2). The actual recombination is thought to occur between Q_A^- and P_{680}^+, with the rate determined by the concentration of P_{680}^+.

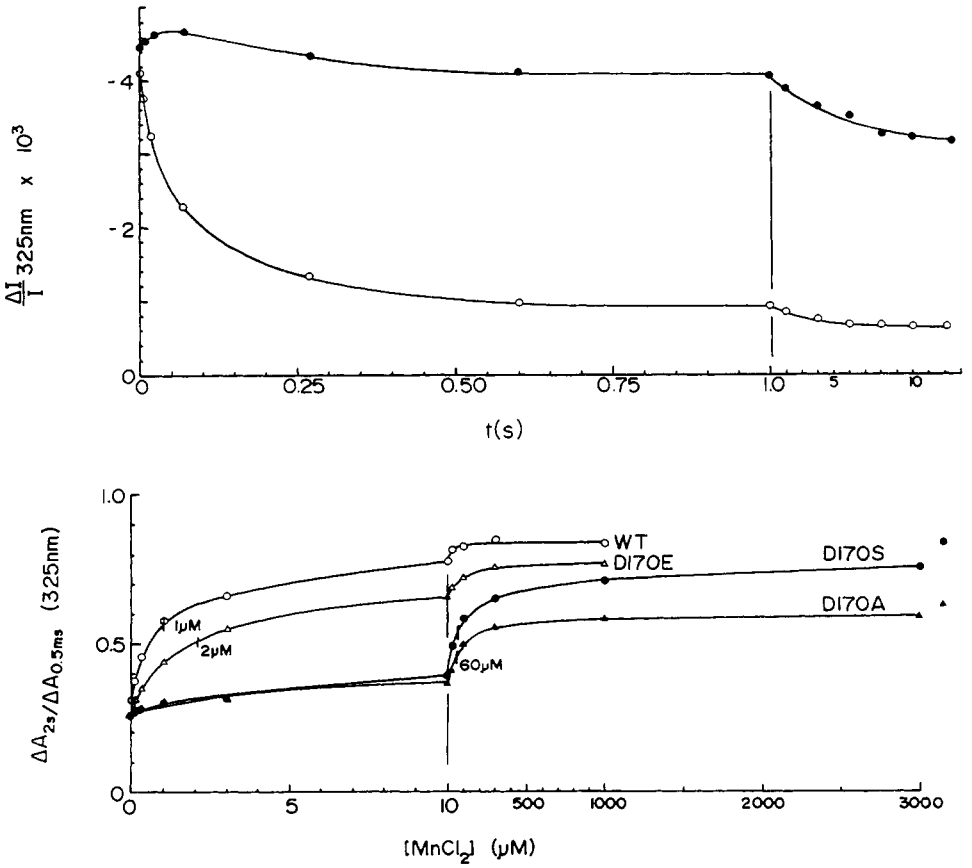

FIGURE 4: (a, top) Kinetics of charge recombination between Q_A^- and the PSII donor side following a single 2-μs flash (≥85% of saturation) excitation of PSII core complexes of WT in the presence and absence of Mn^{2+}. The core complexes (10 μM chlorophyll) were suspended in 20 mM MES-NaOH (pH 5.7), 0.25 mM EDTA, and 0.5 μM $K_3Fe(CN)_6$ with (●) and without (○) 1 mM $MnCl_2$. (b, bottom) The concentration dependence of the ability of Mn^{2+} to block the reoxidation of Q_A^- by charge recombination in WT (○) and mutant core complexes of D1-Asp170Glu (△), D1-Asp170Ser (●), and D1-Asp170Ala (▲) following a single 2-μs flash (≥85% of saturation) excitation. The ordinate scale is the ratio of the concentration of Q_A^- (measured at 325 nm) at 2 s after a flash to that at 0.5 ms. The core complexes (10 μM chlorophyll) were suspended in 20 mM MES-NaOH (pH 5.7), 1 mM $CaCl_2$, and 1 μM $K_3Fe(CN)_6$.

The concentration of P_{680}^+ is in turn determined by the equilibrium

$$S_1Y_ZP_{680}^+ \overset{K_{ZP}}{\rightleftharpoons} S_1Y_Z^+P_{680} \overset{K_{SZ}}{\rightleftharpoons} S_2Y_ZP_{680}$$

with an equilibrium constant $K_{ZP}K_{SZ}$ of approximately 1000. The time taken for the donors to become equilibrated (≤1 ms) is far more rapid than the rate of charge recombination. If the OEC is absent or cannot advance to the S_2 state, then the equilibrium concentration of P_{680}^+ should be higher and determined by K_{ZP} alone, giving rise to an enhanced rate of oxidation of Q_A^- by charge recombination. Such a situation is actually observed for Asp170Ser, as shown in Figure 2. Reoxidation of Q_A^- by charge recombination in whole cells of this mutant occurs with a $t_{1/2}$ of 15–20 ms, 50 times more rapid than in WT cells (Figure 2), but 20 times slower than recombination between Q_A^- and P_{680}^+, as measured in PSII core complexes [$t_{1/2}$ = 1 ms (Metz et al., 1989)]. The accelerated charge recombination in Asp170Ser is not due to an alteration of K_{ZP} as the rate of charge recombination between Q_A^- and Y_z^+ is similar in core complexes isolated from WT and mutant strains (Figure 3). We conclude that, in the D1-Asp170Ser mutant, Y_z is the terminal electron donor owing to the absence or blockage of the OEC.

The Asp170Glu mutant consistently shows a phase of charge recombination (Figure 2), slower still than that of WT, implying that the S_2 state is somewhat more stable than that of WT. This observation is consistent with an enhanced S_2 state lifetime (Table I), measured according to Forbush et al. (1971). Three saturating light flashes were given to previously dark-adapted whole cells. O_2 evolution was measured on the third flash as a function of the time interval between the first flash (which generates S_2) and the second flash (which advances the remaining S_2 to S_3).

Those mutants capable of some light-saturated oxygen evolution show fast phases of charge recombination in whole cells (measured as in Figure 2) intermediate in amplitude between that observed for WT and D1-Asp170Ser. In these mutants, those centers still capable of O_2 evolution show S_2 state lifetimes similar to those observed for Asp170Glu (Table I). There is therefore a heterogeneity in the PSII centers, those with an intact OEC and those that are impaired. Those with an intact OEC show somewhat altered redox properties of the S_2 state giving an equilibrium constant, K_{SZ}, 1.5–3-fold higher than that in WT.

Mutation at Asp170 Affects Mn^{2+} Oxidation. So far the results obtained would be consistent with three possible hypotheses for the nature of the lesion induced by mutation at Asp170: (1) a loss of a Ca^{2+}-binding site, the presence of which is required for the normal formation of state S_2 (Kalosaka et al., 1990), (2) an increase in the E_m of S_2/S_1 also blocking S_2 formation, and (3) an inability to bind Mn^{2+} and to assemble the OEC. While all of these remain possibilities, the experiments that follow support the third hypothesis.

Hoganson et al. (1989) have shown in PSII membranes from spinach that exogenous Mn^{2+} acts as an electron donor to Y_z^+. Core complexes lacking both endogenous manganese and Q_B were isolated from WT and from the D1-Asp170

mutants, and the reduction of Y_z^+ by exogenous Mn^{2+} was examined in two ways: (1) by the ability of Mn^{2+} to block reoxidation of Q_A^- by recombination with the electron donor side and (2) by the dependence of the rate of reduction of Y_z^+ on the concentration of Mn^{2+}.

Figure 4a shows the rate of reoxidation of Q_A^- in the WT core complex in the presence of either 0.25 mM EDTA or 0.25 mM EDTA plus 1 mM Mn^{2+}. In the absence of added Mn^{2+}, Q_A^- is reoxidized with a $t_{1/2}$ of ~70 ms at pH 5.7. We have previously shown that in such complexes Y_z^+ is reduced with the same kinetics (Metz et al., 1989), indicating that Y_z^+ is the ultimate recipient of the returning electron. With the addition of Mn^{2+}, the reoxidation of Q_A^- by recombination is largely blocked, consistent with the reduction of Y_z^+ by Mn^{2+}. The concentration of Mn^{2+} necessary to block half of the centers from recombining gives the K_m for Y_z^+ reduction by exogenous Mn^{2+}. Such an experiment is shown in Figure 4b for a number of D1-170 mutants. The K_m obtained for WT and for the D1-170 mutant strains are shown in Table I and range from 1 to 60 μM. The general trend for the K_m, as for the light-saturated rates of O_2 evolution, approximately follows the pK_a of the amino acid residue at D1-170. The lower the pK_a, the lower the K_m for the reduction of Y_z^+ by Mn^{2+} and the higher the rate of O_2 evolution. The contrast between Ser and Cys and between Asp and Asn would appear to support the idea that it is the chemical properties of the side chain that is critical as each pair has similar geometry and molecular volume. Furthermore, that both Asp170His and Asp170Cys show a low K_m for Mn^{2+} reduction of Y_z^+ would argue against a nonspecific Coulombic effect of the Asp carboxyl group on the binding of Mn^{2+} [see, for example, Linse et al. (1991) for the Ca^{2+}-binding protein, calbindin]. In mutations at nearby sites, replacement of D1 residues Asn165, Ser167, and Ser169 with Ala showed little effect on electron donation to Y_z^+ and on photoautotrophic growth (not shown), in contrast with the same substitution at the Asp170 site. The consequences of the Asp170 substitution would appear to have more to do with a loss of coordinating function than with an alteration in structure or polarity.

The rate of reduction of Y_z^+ was also examined by direct spectroscopic measurement ($\Delta I/I_{429nm} - \Delta I/I_{436nm}$) following a single exciting flash in PSII WT core complexes as a function of the exogenous Mn^{2+} concentration. These wavelengths were chosen to minimize the contributions from P_{680}^+–P_{680} and Q_A^-–Q_A. The results (not shown) indicate that, as the concentration of exogenous Mn^{2+} increases up to 10 μM, there is an increase in the amplitude of a fast component with a constant $t_{1/2}$ of 2–3 ms. This first-order behavior implies that Mn^{2+} binds in the dark to the core complex at a site near Y_z and is oxidized to Mn^{3+} by Y_z^+. Titration of this fast component gives a dissociation constant, K_d, of 1 μM for WT, similar to the K_m reported above. At higher concentrations, a second-order component becomes apparent with a second-order rate constant of 5×10^7 M^{-1} s^{-1}, indicating an additional diffusional process. A similar experiment performed on the Asp170Ser mutant (not shown) shows only second-order behavior, with a second-order rate constant of 5×10^5 M^{-1} s^{-1}. Together, this and the recombination experiment show that the acceleration of the reduction of Y_z^+ by Mn^{2+} is not due to an enhancement of charge recombination but to a true reduction by Mn^{2+}.

Conclusion

In conclusion, we find that D1-Asp170 is critical to oxygen evolution and to donation by Mn^{2+} to the oxidized secondary electron donor, Y_z^+. This work is the first localization of such a site and provides the strongest evidence to date for the participation of reaction center polypeptide D1 in the coordination of manganese. The K_m measured for Mn^{2+} reduction of Y_z^+ in WT core complexes is close to that measured for the first step in the light-driven assembly of the manganese cluster responsible for O_2 evolution [$\leq$1 μM (Blubaugh & Cheniae (1990a)]. That this K_m is greatly elevated by mutations at D1-170 implies that Asp170 probably participates in the first step of assembly of the manganese cluster and is likely to be a ligand to Mn^{2+} at this stage. This role for Asp170 is also consistent with its location only nine residues away from Y_z (D1-Tyr161), the immediate oxidant of the OEC.

Those mutants that are totally inactive for O_2 evolution are probably unable to bind Mn^{2+} sufficiently tightly at the ambient Mn^{2+} concentration of the cell to allow the coordinated assembly of the complex. Those mutants showing some oxygen-evolving activity have sufficient affinity at the first step of assembly to allow complete formation of the cluster in a fraction of the centers. To explain why if some centers can assemble all do not, we would have to postulate the existence of another reaction which competes with the photoactivation process. A likely competing reaction is the photooxidation of another species in competition with that of Mn^{2+}. It has been shown that, following extraction of the Mn cluster with NH_2OH, weak illumination results in the formation of a narrow 10–11 G EPR signal probably arising from a photooxidized chlorophyll other than P_{680}, whose appearance correlates with the loss of the photooxidizability of Y_z and the loss of the ability to reassemble the OEC (Blubaugh & Cheniae 1990b; Ono & Inoue, 1991; Blubaugh et al., 1991). Similar behavior would probably occur in those centers that do not succeed in ligating and photooxidizing Mn^{2+}, resulting in a mix of donor-side inactivated and oxygen-competent centers.

The question as to whether D1-Asp170 is a ligand to the final cluster is still an open one. The lifetime of the S_2 state is affected only slightly by the amino acid present at D1-170, increasing only by a factor less than or equal to 3 with respect to WT. That those mutants that are still partially active for O_2 evolution show (Table I) an oscillation of period 4 in the oxygen flash yields (Joliot & Kok, 1975; Joliot et al., 1969) argues that the turnover of the OEC in the fraction of centers still active is largely unimpaired. We conclude that Asp170 is probably a ligand to Mn at the early stage of assembly of the OEC and, if a ligand to the final cluster, at a position where ligand substitution has only a minor influence on the redox properties of the S states of the OEC. Further EPR or X-ray absorption studies on mutants such as D1-Asp170His or Cys may provide some resolution of this question by showing, respectively, evidence for new nitrogen or sulfur ligation to the Mn cluster.

Acknowledgments

We gratefully acknowledge fruitful discussions with Drs. Gary Brudvig, Richard Debus, Vincent Pecoraro, Joseph Farchaus, and Jeffrey Trost. We thank Eric LaGaccia for excellent technical assistance in the preparation of the PSII core complexes.

Registry No. O_2, 7782-44-7; Asp, 56-84-8; Mn, 7439-96-5.

References

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H., & Rees, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8487–8491.

Babcock, G. T., Blankenship, R. E., & Sauer, K. (1976) *FEBS Lett. 61*, 286–289.

Babcock, G. T., Barry, B. A., Debus, R. J., Hoganson, C. W., Atamian, M., McIntosh, L., Sithole, I., & Yocum, C. F. (1989) *Biochemistry 28*, 9557–9565.

Blubaugh, D. J., & Cheniae, G. M. (1990a) *Plant Physiol. (Suppl.) 93*, 120a.

Blubaugh, D. J., & Cheniae, G. M. (1990b) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 503–506, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Blubaugh, D. J., Atamian, M., Babcock, G. T., Golbeck, J. H., & Cheniae, G. M. (1991) *Biochemistry 30*, 7586–7597.

Brettel, K., Schlodder, E., & Witt, H. T. (1984) *Biochim. Biophys. Acta 766*, 403–415.

Butler, W. L., Visser, J. W. M., & Simons, H. L. (1973) *Biochim. Biophys. Acta 292*, 140–151.

Debus, R. J. (1991) *Biochim. Biophys. Acta* (in press).

Debus, R. J., Barry, B. A., Sithole, I., Babcock, G. T., & McIntosh, L. (1988) *Biochemistry 27*, 9071–9074.

DeRose, V. J., Yachandra, V. K., McDermott, A. E., Britt, R. D., Sauer, K., & Klein, M. P. (1991) *Biochemistry 30*, 1335–1341.

Diner, B. A., Reis, D. F., Cohen, B. N., & Metz, J. G. (1988) *J. Biol. Chem. 263*, 8972–8980.

El-Kabbani, O., Chang, C.-H., Tiede, D., Norris, J., & Schiffer, M. (1991) *Biochemistry 30*, 5361–5369.

Forbush, B., Kok, B., & McGloin, M. P. (1971) *Photochem. Photobiol. 14*, 307–321.

George, G. N., Prince, R. C., & Cramer, S. P. (1989) *Science 243*, 789–791.

Gerken, S., Brettel, K., Schlodder, E., & Witt, H. T. (1988) *FEBS Lett. 237*, 69–75.

Hansson, Ö., & Wydrzynski, T. (1990) *Photosynth. Res. 23*, 131–162.

Hoganson, C. W., & Babcock, G. T. (1988) *Biochemistry 27*, 5848–5855.

Hoganson, C. W., Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1989) *Photosynth. Res. 22*, 285–293.

Joliot, A., & Joliot, P. (1964) *C. R. Hebd. Seances Acad. Sci. 258*, 4622–4625.

Joliot, P., & Joliot, A. (1968) *Biochim. Biophys. Acta 153*, 625–634.

Joliot, P., & Kok, B (1975) in *Bioenergetics of Photosynthesis* (Govindjee, Ed.) pp 387–412, Academic Press, New York.

Joliot, P., Barbieri, G., & Chabaud, R. (1969) *Photochem. Photobiol. 10*, 309–329.

Kalosaka, K., Beck, W. F., Brudvig, G., & Cheniae, G. (1990) in *Current Research in Photosynthesis* (Baltcheffsky, M., Ed.) Vol. I, pp 721–724, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475.

Komiya, H., Yeates, Y. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012–9016.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Lichtenthaler, H. K. (1987) *Methods Enzymol. 148*, 350–382.

Linse, S., Johansson, C., Brodin, P., Grundström, T., Drakenberg, T., & Forsén, S. (1991) *Biochemistry 30*, 154–162.

Longhi, R., & Drago, R. S. (1965) *Inorg. Chem. 4*, 11–14.

March, J. (1968) in *Advanced Organic Chemistry, Reactions, Mechanisms & Structure*, pp 220–221, McGraw-Hill, New York.

Marder, J. B., Chapman, D. J., Telfer, A., Nixon, P. J., & Barber, J. (1987) *Plant Mol. Biol. 9*, 325–333.

Mauzerall, D. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1358–1362.

Metz, J. G., Nixon, P. J., Rögner, M., Brudvig, G. W., & Diner, B. A. (1989) *Biochemistry 28*, 6960–6969.

Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1–7.

Nanba, O., & Satoh, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 109–112.

Nixon, P. J., & Diner, B. A. (1990) in *Proceedings of the Twelfth Annual International Conference of the IEEE Engineering in Medicine and Biology Society* (Pedersen, P. C., & Onaral, B., eds.) Vol. 12, pp 1732–1734, IEEE, Piscataway, NJ.

Nixon, P. J., Rögner, M., & Diner, B. A. (1991) *Plant Cell 3*, 383–395.

Ono, T.-A., & Inoue, Y. (1991) *Biochemistry 30*, 6183–6188.

Pakrasi, H., & Vermaas, W. F. J. (1991) in *Topics in Photosynthesis: The Photosystems* (Barber, J., Ed.) Elsevier, Amsterdam.

Penner-Hahn, J. E., Fronko, R. M., Pecararo, V. L., Yocum, C. F., Betts, S. D., & Bowlby, N. R. (1990) *J. Am. Chem. Soc. 112*, 2549–2557.

Ratilla, E. M. A., & Kostic, N. M. (1988) *J. Am. Chem. Soc. 110*, 4427–4428.

Reinman, S., Mathis, P., Conjeaud, H., & Stewart, A. (1981) *Biochim. Biophys. Acta 635*, 429–433.

Robinson, H. H., & Crofts, A. R. (1983) *FEBS Lett. 153*, 221–226.

Sauer, K., Guiles, R. D., McDermott, A. E., Cole, J. L., Yachandra, V. K., Zimmermann, J.-L., Klein, M. P., Dexheimer, S. L., & Britt, R. D. (1988) *Chem. Scr. 28A*, 87–91.

Taylor, M. A., Nixon, P. J., Todd, C. M., Barber, J., & Bowyer, J. R. (1988) *FEBS Lett. 235*, 109–116.

van Best, J. A., & Mathis, P. (1978) *Biochim. Biophys. Acta 503*, 178–188.

van Gorkom, H. J. (1985) *Photosynth. Res. 6*, 97–112.

Wieghardt, K. (1989) *Angew. Chem., Int. Ed. Engl. 28*, 1153–1172.

Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–778.

Yeates, T. O., Komiya, H., Chirino, A., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7993–7997.