Participation of the C-terminal Region of the D1-polypeptide in the First Steps in the Assembly of the Mn₄Ca Cluster of Photosystem II*

Received for publication, June 29, 2006, and in revised form, December 29, 2006 Published, JBC Papers in Press, January 2, 2007, DOI 10.1074/jbc.M606255200

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Amino acid residue D1-Asp 170 of the D1-polypeptide of photosystem II was previously shown to be implicated in the binding and oxidation of the first manganese to be assembled into the Mn₄Ca cluster of the oxygen-evolving complex (OEC). According to recent x-ray crystallographic structures of photosystem II, D1-Glu 333 is proposed to participate with D1-Asp 170 in the coordination of Mn₄ of the OEC. Other residues in the C-terminal region of the D1-polypeptide are proposed to coordinate nearby manganese of the cluster. Site-directed replacements in Synechocystis sp. PCC 6803 at D1-His 332, D1-Glu 333, D1-Asp 342, D1-Ala 344, and D1-Ser 345 were examined with regard to their ability to influence the binding and oxidation of the first manganese in manganese-depleted photosystem II core complexes. Direct and indirect measurements reveal in all mutants, but most marked in D1-Glu 333 replaced by His, an impaired ability of Mn²⁺ to reduce Y₂, indicating a reduced ability (elevated Km) compared with WT to bind and oxidize the first manganese of the OEC. The effect on the Km of these mutations is, however, considerably weaker than some of those constructed at D1-Asp 170 (replacement by Asn, Ala, and Ser). These observations imply that the C-terminal residues ultimately involved in manganese coordination contribute to the high affinity binding at D1-Asp 170 likely through electrostatic interactions. That these residues are far from D1-Asp 170 in the primary structure of the D1-polypeptide, imply that the C terminus of the D1-polypeptide is already close to its mature conformation at the first stages of assembly of the Mn₄Ca cluster.

Photosystem II (PSII) 2 of oxygenic photosynthesis is a multisubunit membrane-associated pigment-protein complex that supports light-driven oxidation of water to molecular oxygen. Recent x-ray crystallographic structures of the PSII core complex have been reported at resolutions ranging from 3.0 to 3.8 Å (1–5). Two of these (3–5) depict in detail the structure and coordination environment of the Mn₄Ca cluster, responsible for water oxidation (oxygen-evolving complex or OEC). The cluster contains four manganese, whose oxidation states are thought to range from II to IV (6), depending on the state of oxidation of the OEC, and one Ca²⁺. The proteinaceous ligands (a total of 11–12 (5)) to the cluster are contributed by two polypeptides, D1 (PsbA) and CP43 (PsbC), with additional coordination provided by water molecules (including hydroxide), mono-, di-, and tri-μ-oxo bridges and Cl⁻ (7–9).

Charge separation is initiated by the reaction center redox components coordinated by polypeptides D1 and D2 (10–12). The oxidizing equivalent is stabilized on ChlP 680 within 6–8 ps (13, 14) and is transferred to redox-active tyrosine Y₆ (D1-Tyr 161) in tens to hundreds of nanoseconds (15, 16). In tens to hundreds of microseconds, the oxidizing equivalent is transferred to the Mn₄Ca cluster (15, 16), with the localization of the cationic charge, depending on the oxidation state of the cluster and still a matter of speculation (9, 17). The reducing equivalent generated in primary charge separation initially resides on a pheophytin (13, 14), and is transferred to the primary quinone electron acceptor, Qₐ, in ~250 ns (18). Subsequent electron transfer to the secondary electron acceptor, Qₐ, occurs in the hundreds of microseconds time scale and is coupled to proton transfer (19).

Whereas there have been considerable advances in the understanding of the structure and coordination of the Mn₄Ca cluster, the x-ray (3–5) and extended x-ray absorption fine structure (EXAFS)-derived (8, 20) structures provide no information as to how the cluster is assembled. Such information has been obtained through examination of the functional consequences of site-directed mutations (21–23). For instance, Ananyev and co-workers (24) have demonstrated using a D2-Y160F mutant (D2-Tyr 160 replaced by Phe) that redox-active tyrosine Y₁₆₆ probably plays an indirect role in accelerating
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photoactivation (light-driven assembly) of the Mn₄Ca cluster. Extensive site-directed mutagenesis and spectroscopic studies have associated D1-Asp¹⁷⁰ more directly with binding of the first manganese involved in the assembly of the OEC (25-28). For example, Ala, Asn, and Ser mutations at this site raise the apparent dissociation constant (Kₐ) 50-60-fold in manganese-depleted PSII core complexes, as measured by blockage by Mn²⁺ of charge recombination between Qₓ and the oxidized donor side (25) and by the concentration dependence of Mn²⁺ on the rate of reduction of Yₓ⁺ (26). D1-Asp¹⁷⁰ is shown in the x-ray crystallographic structure to coordinate Mn₄, making this manganese, therefore, the most likely to be the first one bound in the assembly process. The two most detailed x-ray structures (3-5) depict Mn₄ to be also coordinated by bidentate, bridging Mn₄ and Mn₃. As a consequence of this, involvement of D1 C terminus in first steps of OEC assembly and other coordinating D1-C-terminal carboxylate residues in facilitating the coordination of the first manganese. 

Experimental Procedures

The glucose-tolerant strain of the cyanobacterium Synechocystis sp. PCC 6803 (31) was used for construction of the site-directed mutants described in this paper. All strains were grown on BG-11 medium as described by Williams (31) and Metz et al. (32). Five millimolar glucose was included in the medium to allow the propagation of mutants inactive in PSII. The site-directed mutations were constructed in psbA3 as described by Nixon and Diner (25) using a host strain (TD41) in which the psbA1, -2, and -3 genes had been previously deleted. Initial rates of oxygen evolution were measured on whole cells of Synechocystis sp. PCC 6803 in saturating light (>610 nm) at 25 °C using a thermostated Hansatech oxygen electrode. The cells were suspended in BG-11 medium containing 5 mM glucose to which 0.3 mM 2,6-dichloro-p-benzoquinone and 1 mM K₃Fe(CN)₆ had been added. The concentration of chlorophyll present in the sample was determined spectroscopically by extracting the cell pigments by brief sonication in methanol and using an extinction coefficient of 79.24 ml mg⁻¹ cm⁻¹ at 665 nm (33). Typical initial rates of oxygen evolution were 490 ± 36 μmol of O₂/mg Chl h⁻¹ in strain TC35 (psbA1⁻, psbA2⁺, with a wild-type copy of psbA3).

PSII core complexes were isolated from cells grown in 20-liter clear plastic carboys (Nalgene Labware). The isolation procedure was performed according to the combined methods of Tang and Diner (34) and Rögner et al. (35), in that order and stored at -80 °C until use. The passage of the core complexes through the hydroxylapatite column (SynChrom HAP-3, SynChrom Inc.) resulted in complete extraction of the Mn₄Ca cluster (25).

Optical spectroscopy on PSII core complexes was performed using a flash detection spectrophotometer based on a design by Joliot et al. (36) and described in Metz et al. (32). This instrument has 1-μs time resolution. Actinic flashes were provided either by a xenon flash (EG&G FX-199, 8-mm arc, 2-μs width at half-height) or a Cyanousure LFDL-3 dye laser using sulforhodamine 640 dye (Exciton Inc., 1-μs width at half-height). The kinetics of charge recombination between Qₓ and the oxidized PSII donor side in the presence and absence of MnCl₂ were followed at 325 nm (absorbance maximum of Qₓ⁻ - Qₓ, (37)) as previously described (25, 38), using weak detecting flashes (EG&G FX-199U) rendered monochromatic by a Jobin Yvon HL300 monochromator. The kinetics of Yₓ⁺ reduction in PSII core complexes, measured at different MnCl₂ concentrations, were performed as described by Diner and Nixon (26). All core complexes were suspended at a concentration of 10 μg/ml chlorophyll in 20 mM MES-NaOH (pH 6.1), 1 mM CaCl₂, 1 to 2 μM K₃Fe(CN)₆.

The same instrument was also used for measurements in whole cells of the amplitude of and kinetics of relaxation of the relative quantum yield of chlorophyll fluorescence (25, 38). This kinetic relaxation, following excitation with saturating actinic light flashes (EG&G FX-199), was monitored using weak detecting flashes (EG&G FX-199) rendered monochromatic by the HL300 monochromator at 422 nm. The filter sets used for the actinic flash and to protect the sample photodiode were the same as used previously (32) except that the Schott LF550 was replaced by a Schott LF470. The same technique was used to determine the relative PSII reaction center concentration per cell, which is proportional to the total variable fluorescence yield at a fixed cell concentration. The variable fluorescence is the difference in the fluorescence yield measured at a given time point after actinic illumination minus the F₀ level (Qₓ oxidized) measured before actinic illumination. Prior to measurements of the evolution of the variable fluorescence following actinic flash illumination, Synechocystis cells at an optical density of 0.9 cm⁻¹ at 730 nm were preincubated for 10 min in BG-11 plus 50 mM HEPES-NaOH (pH 7.5), 0.3 mM p-benzoquinone, and 0.3 mM K₃Fe(CN)₆. The preincubation in the dark was long enough to allow for complete oxidation of the primary and secondary quinone electron acceptors, Qₓ and Qₓ⁻. To measure the kinetics of charge recombination between Qₓ and the oxidized...
donor side, 40 μM DCMU was added after the 10-min dark incubation, blocking oxidation of QA by QA. A single saturating actinic flash was given ≥1 min later and the relaxation of the variable fluorescence yield followed as above. Further addition of 20 mM NH₂OH provides a stable donor electron that acts in place of the Mn₄Ca cluster, blocking charge recombination. A flash of 20 saturating flashes (18 Hz) then results in the stable accumulation of QA. The variable fluorescence yield measured under these conditions is a measure of the relative amounts of PSII on a per cell basis (39).

### RESULTS

**Physiological Characterization of Mutations**—Site-directed mutant strains were constructed in *psbA3* in the *Synechocystis* sp. PCC 6803 host strain TD41, as described by Nixon and Diner (25), and contained one of each of the following mutations: D1-His332 was replaced by Leu; D1-Glu333 by Ala, Cys, Gln, and His; D1-Asp342 by Val; D1-Ala344 by a Stop codon; and D1-Asp345 by Pro. All of these strains lack PsbA1 and PsbA2. The wild type strains (TC31 and TC31, to which the mutant strains are compared, contain wild-type *PsbA3* but lack PsbA1 and -2. TC31 and TC35 were derived from identical constructs and host strains. The site-directed mutants show depressed rates of oxygen evolution (Table 1) and are unable to grow photoautotrophically, with the exception of D1-E333Q that is very weakly photoautotrophic (this work and Ref. 40). In addition, the amounts of PSII core complex isolated biochemically from the D1-E333H, Ala, and Cys mutants were significantly lower than those determined using 20 mM NH₂OH plus DCMU (Table 1). The consequent long-lived excited states, which reside for the most part in the antenna, are responsible for the elevated fluorescence yield. The state P₆₈₀⁺F₆₄₇⁻ QA formed following primary and secondary charge separation of photoexcited state P₆₈₀⁺F₆₄₇⁻ QA is very much lower in energy than P₆₈₀⁺F₆₄₇⁻ QA and has a fluorescence yield close to F₁. This is because P₆₈₀⁺F₆₄₇⁻ QA quenches the fluorescence (41). Upon reduction of P₆₈₀⁺F₆₄₇⁻ QA, but prior to the oxidation of QA, the fluorescence yield increases to F₂ (42). Upon electron transfer from QA to QB, the fluorescence yield again decreases, with the rate of decrease tracking the kinetics of electron transfer between the quinones. In *Synechocystis* cells and under the conditions measured here, the relationship between the quantum yield of fluorescence (between F₁ and F₂) and the relative concentration of QA is to a first approximation linear.

Monitoring the variable fluorescence yield following each of a series of saturating flashes provides a measure of the relative concentration of PSII and of the intactness of the electron donor side of the PSII reaction center. The relative concentration of the PSII centers in the various mutants was obtained by pretreating cells with 0.3 mM p-benzoquinone and 0.3 mM K₃Fe(CN)₆ followed by illumination in the presence of 40 μM DCMU and 20 mM NH₂OH, as described under “Experimental Procedures.” The results are shown in Table 1 and show a substantial difference in the center concentration between the different D1-Glu332 mutants. Fig. 1A shows the variation in the quantum yield of fluorescence following each of a series (1.67 Hz) of five saturating actinic flashes given to whole cells of WT (TC35) *Synechocystis* sp. PCC 6803, pretreated with 0.3 μM ferricyanide and 0.3 μM p-benzoquinone in BG-11 medium plus 50 mM HEPES-NaOH (pH 7.5). The presence of these oxidants assures that the acceptor side of the reaction center is initially in the QAQB state in the dark. The amplitude of the variable fluorescence yield measured at 100 μs after the first actinic flash for the Gln, His, and Cys mutants was determined to be 0.89, 0.45, and 0.40, respectively, compared with WT (TC35). The relative amplitudes of the variable fluorescence observed in Fig. 1 in the mutants were somewhat greater than those determined using NH₂OH plus DCMU (Table 1) for reasons that may have to do with the influence of the intactness of the Mn₄Ca cluster on the

### TABLE 1

**Comparison of WT and site-directed mutants**

| Strain           | Photoautotrophic growth[a] | Cells grown at 50 μE/m²s (2 μE/m²s) | O₂ rate per chlorophyll[b] % of WT | Relative PSII centers per cell[c] | Kₘ-overall[d] μM | Kₘ QA/Kₘ QA⁺ [% of each component] |
|------------------|-----------------------------|--------------------------------------|-----------------------------------|----------------------------------|----------------|-----------------------------------|
| WT (TC31, TC35)  | +                           |                                      | 100 (100)                         | 100 (100)                        | 0.3            | 0.044 (57%); 9.8 (43%)            |
| D1-H322L        | -                           |                                      | 0                                 | 36                               | 1.9            | 1.9 (90%); 9.8 (43%)             |
| D1-E333Q        | + (weak)                    |                                      | 29 (16)                           | 60 (43)                          | 2.5            | 0.16 (27%); 4.8 (73%)            |
| D1-E333H        | ≤7 (≤5)                     |                                      | 27 (9)                            | 20                               | 0.035 (29%); 44 (71%) |
| D1-E333A        | -                           |                                      | ND[d]                             | ND                               | ND             | ND                               |
| D1-E333C        | ≤6 (0)                      |                                      | 10 (10)                           | ND                               | ND             | ND                               |
| D1-D344V        | -                           |                                      | 16                               | 3.0                              | 0.005 (11%); 3.5 (89%) |
| D1-A344stop     | -                           |                                      | 0                                | 40–57                            | 3.4            | 4.3 (83%)                        |
| D1-S345P        | -                           |                                      | 20–25                            | 1.7                              | 0.15 (34%); 4.1 (66%)       |

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[a] Determined in liquid growth medium or Petri plates in the absence of glucose.
[b] Initial O₂ rates measured in saturating light.
[c] Estimated from the total yield of variable chlorophyll fluorescence (F₀-Fₑ)max.
[d] Kₘ QA/Kₘ QA⁺ are apparent dissociation constants for Mn²⁺ in two-component hyperbolic fits of the charge recombination data (see Fig. 4).
QA/QA reduction potential (43), which influences the kinetics of QA oxidation. The variable fluorescence and the yields of PSII core complexes were consistently higher in the case of D1-E333Q than for D1-E333H and Cys for reasons that likely have to do with Gln being a better steric match to the wild-type Glu.

The decay of the fluorescence yield, following each actinic flash in Fig. 1A, reflects the oxidation of QA by QB in WT (TC35). These kinetic traces show a marked oscillation of period two, rapid on odd-numbered flashes and slow on even-numbered flashes. On odd-numbered flashes, QAQB is generated and rapidly transfers an electron to QB forming QAQB (reaction 1). QAQB is then generated on even-numbered flashes. Electron transfer from QAQB to form QAQBH+ (reaction 2) is energetically unfavorable and QA oxidation must be coupled to the protonation of QB (44). Electron transfer to form QAQBH+ is therefore slowed relative to reaction 1 by the need to protonate the secondary quinone prior to or concomitant with the electron transfer (44, 45).

Fig. 1B shows an experiment similar to that of Fig. 1A for whole cells of the three D1–333 mutants treated as above. No period two oscillations of the variable fluorescence yield are apparent. In addition, the fluorescence yield at the earliest time point (100 μs) progressively decreases with actinic flash number. This observation is in contrast to that of WT (TC35) where the initial fluorescence yields, following each actinic flash, show a damped period two oscillation around a horizontal straight line. A progressively increased quenching of fluorescence at 100 μs, accompanied by an absence of period two oscillations, indicates the absence of or impairment of tertiary electron donation. In such a case, YZ is unable to be fully reduced in the time between a first actinic light flash and a second. As a result of the incomplete reduction of YZ, the lifetime of P680 is increased, generating increased amounts of P680PheoQA at 100 μs following each subsequent actinic flash of the flash series. Because P680+ is a quencher of fluorescence (see above and Ref. 41), the increased concentration of P680PheoQA results in a progressive quenching. The quenching is already apparent on the second flash and more marked on the third and subsequent flashes. It is more extreme for the D1-E333H and Cys mutants, similar to what was previously observed for the most perturbed mutations constructed at D1–170 (e.g. D1-D170S and Ala (25)) and consistent with their reduced ability to evolve O₂. The quenching is less marked for the D1-E333Q mutant, which is less impaired in its ability to evolve O₂. These observations are similar to those reported by Chu et al. (40) for the His and Gln mutants and indicate either an impaired ability of the OEC to advance to the higher S states or a lack of assembly of the manganese cluster in some (Gln mutant) or nearly all centers (His, Cys, and Ala mutants).
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Kinetics of Charge Recombination—Charge recombination in PSII between the reduced acceptor and oxidized donor sides is thought to occur through P680+. A consequence of the increased lifetime of YZ′ is that there should be an increased equilibrated concentration of \( P_{680+} \), which means that the mutants should show an increase in the rate of charge recombination between QA and the oxidized donor side. The kinetics of QA oxidation by charge recombination can be followed by measuring the decay of the quantum yield of fluorescence in the presence of 40 \( \mu M \) DCMU, which blocks forward oxidation of QA. Fig. 2 shows such a charge recombination experiment in whole cells of WT (TC35) and D1-E333Q. The kinetics were fit with two exponential terms plus a constant. Of the two exponential components obtained for the WT (TC35), the slower and major component gave a \( \tau \) of 1.89 s (\( t_{1/2} = 1.31 \) s, 65% of total), whereas the faster and minor component gave a \( \tau \) of 141 ms (\( t_{1/2} = 98 \) ms, 23% of total). Of the two exponential components for D1-E333Q the faster and major component gave a \( \tau \) of 51 ms (\( t_{1/2} = 35 \) ms, 45% of total) and a slower and minor component gave a \( \tau \) of 2.63 s (\( t_{1/2} = 1.82 \) s, 37% of total). The large slow component observed in the WT (TC35) reflects the recombination between QA and the S2 state of the OEC. The smaller percentage slow component in the D1-E333Q mutant likely represents the reduced fraction of centers that have also generated \( 2Q_A^- \) with an assembled MnCa cluster. The larger faster component in the D1-E333Q mutant is consistent with an increased lifetime of YZ′ in those centers in which the MnCa cluster is not assembled and which have not been able to oxidize a mononuclear Mn\(^{2+}\) during the lifetime of the charge separated state. The increased YZ′ concentration in turn produces an increased equilibrated concentration of \( P_{680+} \), accelerating the recombination.

Mn\(^{2+}\) Influence on Charge Recombination of QA with YZ′—One reason for an increased lifetime of YZ′ in the D1-E333Q mutant is the partial absence of or inability to oxidize manganese, the tertiary electron donor of PSII. Measurements of the kinetics of charge recombination in the presence of varying concentrations of MnCl\(_2\) provide a convenient means to evaluate the ability to bind and oxidize Mn\(^{2+}\) in PSII core complexes that had been previously depleted of manganese. In such complexes, the extent of re-oxidation of QA by charge recombination in the presence of Mn\(^{2+}\) monitors how effectively Mn\(^{2+}\) donates to YZ′ during the lifetime of the charge separated state (25). In the absence of Mn\(^{2+}\), QA is re-oxidized by recombination through P680 with YZ′. Forward electron transfer does not occur from QA due to the absence of QA in the PSII core complexes. In the presence of increasing concentrations of Mn\(^{2+}\), the re-oxidation of QA by recombination is increasingly blocked as YZ′ is reduced by Mn\(^{2+}\) faster than it can be reduced by back reaction with QA. The reason for the blockage is that Mn\(^{3+}\) is much more stable than YZ′ with regard to charge recombination. An example of how this works is shown in Fig. 3 where the lifetime of QA is followed at 325 nm, an absorbance maximum of the QA–QA difference spectrum (37). Here we compare WT (TC35) manganese-depleted PSII core complexes in the presence of 0.25 mM EDTA, 1 \( \mu M \) \( K_3Fe(CN)_6 \), and 1 mM CaCl\(_2\) in the presence or absence of 1 mM exogenous Mn\(^{2+}\) (MnCl\(_2\)). One micromolar K\(_3Fe(CN)_6\) is added to assure that QA is initially oxidized but not so concentrated as to oxidize QA in competition with charge recombination. In the absence of Mn\(^{2+}\), reoxidation of QA is rapid and markedly biphasic (\( t_{1/2} = 53 \) ms (45%) and 2.6 s (24%)), with the rapid component arising from YZ′QA′ recombination. In the presence of 0.75 mM Mn\(^{2+}\) (not complexed by EDTA) the reoxidation of QA by recombination with YZ′ is largely blocked due to YZ′ reduction by Mn\(^{2+}\) with exponential components with \( t_{1/2} = 98 \) ms (9%) and 4.8 s (57%). The slow phase of QA oxidation likely comes from slow electron transfer to \( K_3Fe(CN)_6 \) or recombination with Mn\(^{3+}\) or a combination of the two.

To evaluate the efficacy of electron donation by Mn\(^{2+}\) to YZ′, the ratio of \( \Delta I/\Delta I_0 \) at 2 s divided by \( \Delta I/\Delta I_0 \) at 500 \( \mu \)s was calculated as
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FIGURE 4. The concentration dependence of the ability of MnCl₂ to block the reoxidation of Q₀ by charge recombination in WT (TC31) (■) and mutant core complexes of D1-E333Q (●, red), D1-E333H (△, blue), D1-H332L (◇, pink), D1-D342V (□, orange), D1-A344Stop (●, green), and D1-S345P (◇, purple) following a 2-μs saturating flash. The ordinate scale is the ratio of the ΔI/I of Q₅ − Q₀ (measured at 325 nm) at 2 s after the actinic flash to that at 0.5 ms after the actinic flash. The mutant plots were normalized to the same limits as the wild-type (TC31). The core complexes (10 μg/ml chlorophyll) were suspended in 20 mM MES-NaOH (pH 6.1), 1 mM CaCl₂, and 1 μM K₃Fe(CN)₆.

in Ref. 25 using manganese-depleted PSII core complexes. In the absence of added Mn²⁺, this ratio is low (typically 0.35–0.4). The ratio increases with increasing concentrations of Mn²⁺, as recombination is increasingly blocked by Mn²⁺ reduction of Yₐ. By plotting the ratio as a function of the Mn²⁺ concentration (Fig. 4), we can evaluate how well Mn²⁺ is bound by the core complex and oxidized by Yₐ during the lifetime of the charge separated state. The Mn²⁺ titrations in the wild-type and site-directed mutants were fit with two hyperbolic components (Y = A[Mn²⁺]/([Mn²⁺] + Kₘ₁) + B[Mn²⁺]/([Mn²⁺] + Kₘ₂) + C), where Kₘ₁ and Kₘ₂ are apparent dissociation constants for the binding and oxidation of Mn²⁺. Table 1 records both the concentrations of MnCl₂ to block half of the centers overall from recombining (Kₘ,overall) and the Kₘ values obtained from the bi-hyperbolic fits. It is understood that the Kₘ is the concentration of Mn²⁺ that blocks half of the centers showing that hyperbolic component. The dependence of the blockage of charge recombination on [Mn²⁺] is clearly different between the WT (TC31) and various mutant complexes. These include, in addition to the D1-Glu³³³ mutants, D1-H332L (disruption of coordination to Mn1 or Mn2), D1-D342V (disruption of coordination to Mn1 and Mn2), D1-A344Stop (disruption of coordination to Mn2 and Ca²⁺) and D1-S345P (disruption of coordination to Mn2 and Ca²⁺), through an inability to proteolytically process the D1 C terminus (46). The proposed disruptions of coordination are based on the models of Ferreira et al. (4) and Löll et al. (5). The WT (TC31) shows blockage of charge recombination in half of the centers (Kₘ,overall) at a MnCl₂ concentration of 0.3 μM (Table 1). Overall half-blockage for the mutants range from 1.3 (D1-H332L) to 20 μM MnCl₂ (D1-E333H). D1-E333Q, D1-D342V, D1-A344Stop, and D1-S345P show intermediate values of 2.5, 3.0, 3.4, and 1.7 μM, respectively. A fit of the titration to two hyperbolic components (Table 1) gives components of 0.044 (57%) and 9.8 (43%) μM for the WT (TC31). In the case of the site-directed mutants, the dominant component is in the micromolar range with the exception of D1-E333H, which has a major component of 44 μM. The submicromolar components are all of much smaller amplitude in the mutants than they are in the WT. All of the mutants clearly require higher concentrations than WT (TC31) of MnCl₂ to block charge recombination, although in detail their dependence on the Mn²⁺ concentration does differ in each case. The elevated Kₘ values reflect the contributions that all of these sites make to the coordination and oxidation of Mn²⁺ at the D1-Asp³⁷⁶ site, consistent with their localization in close proximity to the high affinity binding site. None of the constructed mutants produce effects of the same magnitude as some of those at D1-Asp³⁷⁶ (e.g. replacement by Asn, Ala, and Ser), implying that the contributions of the wild-type residues to the binding and oxidation of the first Mn²⁺ are indirect, likely through electrostatic effects that enhance the local concentration of Mn²⁺. The range in Kₘ between the mutants would then likely reflect differences in electrostatic charge and localization.

Influence of Mn²⁺ on the Kinetics of Reduction of Tyrosine Yₐ—The titration of the blocking of charge recombination by MnCl₂ is an indirect measure of the rate of reduction of Yₐ by Mn²⁺, as it represents the competition between two pathways for Yₐ reduction (from Mn²⁺ and Q₅). Although much more costly in core complexes, it is possible to measure the Yₐ lifetime directly following a saturating actinic flash by measuring the absorbance difference at 429 – 436 nm as a function of time (26). This difference is primarily due to Yₐ − Yₐ, and comes from electrostatic field changes associated with Yₐ oxidation on the spectrum of ChlP₉₁ (47). By taking this difference, one also eliminates the absorbance difference contributed by P₆₈₀ − P₆₈₀. There is also a contribution from Q₅ − Q₅, which was eliminated by measuring the kinetics of Q₅ oxidation at 325 nm and multiplying by a correction factor of 0.45, a reflection of the extinction coefficient at 325 nm versus that at 429 – 436 nm. This value was then subtracted from the 429 – 436 nm difference to obtain the absorbance difference of Yₐ − Yₐ only. An example is shown in Fig. 5, which plots the kinetics of relaxation of Yₐ − Yₐ following a single saturating actinic flash in core complexes of WT (TC31) and D1-E333Q in the absence and presence of 1, 3, and 10 μM MnCl₂. The pseudo first-order rate constants were extracted from fits to a family of experiments of this type performed on both WT (TC31 and TC35) and the D1-E333Q mutant. In an earlier study on WT (TC31) (26), the data were fit to 1 s with two exponentials plus a constant. In the present study the data were fit out to 170 ms with a single exponential plus a constant, providing a greater constraint on the fitting. The faster of the two rate constants obtained in the earlier study and the single rate constant in the present study are within a factor of two of each other.

Fig. 6 shows the pseudo first-order rate constants associated with the reduction of Yₐ plotted as a function of the concentration of MnCl₂ in core complexes isolated from WT (both TC35 and TC31) and mutants D1-D170S and D1-E333Q. In the absence of added MnCl₂, the WT strains...
show one rate for core complexes prepared as described under “Experimental Procedures” (62 s^{-1}, 73% of the total relaxation) and another much slower component (4 s^{-1}) in core complexes prepared in the same way, but washed with 2.5 mM EDTA (pH 8.0). The EDTA treatment removes tightly bound adventitious Mn^{2+} that is capable of reducing Y_{Z^2}. In the absence of a tertiary electron donor, the rate of 4 s^{-1} is dominated by charge recombination between Q_A and Y_{Z^2}, considerably faster than the rate (≈0.14 s^{-1}) of Q_A oxidation by K_Fe(CN)_6 as mentioned above. In the case of the core complexes from the D1-D170S mutant, such EDTA washing is unnecessary to observe the slow rate of Y_{Z^2} reduction in the absence of added Mn^{2+} (1.4 s^{-1}, 80% of the total relaxation), indicating the importance of D1-Asp^{170} in the coordination of Mn^{2+} in this binding site. The case of the D1-E333Q mutant is intermediate between the two showing a rate of Y_{Z^2} reduction in the absence of Mn^{2+} of 45 s^{-1} (39% of the total relaxation).

The rate of Y_{Z^2} reduction in WT (TC31) was shown previously (26) to increase linearly with MnCl_2 up to a concentration of 30 \mu M. Fig. 6 shows here for both TC31 and TC35 a second-order rate constant of 2.6 \times 10^7 M^{-1} s^{-1} for Mn^{2+} reduction of Y_{Z^2}. As shown previously (26), the linear increase in the rate of reduction of Y_{Z^2} with [Mn^{2+}] in the D1-D170S mutant shows a much smaller second-order rate constant, here 8.0 \times 10^5 M^{-1} s^{-1}, indicative of a much weaker binding of Mn^{2+}. In the case of the D1-E333Q mutant, the dependence on [Mn^{2+}] is different from the other two cases, showing a generally sigmoidal dependence with small effects on rate at 1 and 3 \mu M MnCl_2. The rates and percent contribution obtained from the fits for the D1-E333Q mutant in the presence of 0, 1, 3, and 10 \mu M MnCl_2 are 45 (39%), 47 (76%), 71 (75%), and 358 s^{-1} (77%), respectively. Like the initial observation attributed to adventitious Mn^{2+} binding (in the absence of added MnCl_2), the D1-E333Q mutant shows an intermediate dependence on [Mn^{2+}], weaker than WT and stronger than that of D1-D170S. This dependence mirrors that of the effect of Mn^{2+} on charge recombination (Fig. 4). Both sets of experiments reflect a weakening of Mn^{2+} binding and oxidation in the D1-Glu^{333} mutants, consistent with participation of D1-Glu^{333} and the other C-terminal coordinating residues in the binding and oxidation of Mn^{2+} in the earliest steps of assembly of the Mn4Ca cluster but at a level substantially weaker than that attributed to D1-Asp^{170}.

**DISCUSSION**

We have previously shown that D1-Asp^{170} is implicated in binding and oxidation of the first manganese of the OEC. Mutations at this site not only affect the affinity of Mn^{2+} for the reaction center, but they affect as well the coordination envi-
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Environment of the Mn\(^{3+}\) or Mn\(^{4+}\) (depending on the mutation) generated upon illumination at a concentration of 1.2 Mn\(^{2+}\)/center (28). The 3.0- (5) and 3.5-Å (4) x-ray crystallographic structures of PSII depict D1-Asp\(^{170}\) as a ligand in the fully assembled manganese complex, coordinating Mn\(^4\), implying that this manganese is the first to be assembled into the Mn\(_{6}\)Ca cluster. Also depicted coordinating this manganese is D1-Glu\(^{333}\), a residue located far from D1-Asp\(^{170}\) in the primary structure of the D1-polypeptide. The x-ray structure shows the C-terminal region of the D1-polypeptide to be folded in such a way as to bring these two residues together to permit simultaneous coordination of the same manganese. Even if there were some error in the x-ray structures regarding the details of cluster co-ordination (see Introduction and below), it is certain that these residues must be in close proximity. The experiments presented here allow us to distinguish between two cases: 1) where the conformation of the C-terminal region is already close to its mature form prior to or during the binding and oxidation of Mn\(^{2+}\), bringing D1-Asp\(^{170}\) and some D1 C-terminal residues close enough to cooperatively contribute to the binding and oxidation of the first manganese, and 2) where the conformation of the C-terminal region is established following the binding and oxidation of one or more manganese and where the D1 C-terminal residues do not contribute at all to the conformation of the first Mn.

PSII Donor Side Electron Transfer and Charge Recombination—Examination of site-directed mutations constructed at D1–333 to replace Glu with Gln, His, and Cys and at other sites in the C-terminal region, allows one to discriminate between these possibilities. Experiments in whole cells of the D1-Glu\(^{333}\) mutants show different levels of impairment in oxygen evolution relative to WT (Table 1), a progressive fluorescence quenching observed following each of a series of five actinic flashes and a loss of the oscillation of period two in the kinetic relaxation following each actinic flash (Fig. 1). These observations imply a loss of tertiary electron donation in PSII, consistent either with a reduced affinity for Mn\(^{2+}\) binding and oxidation resulting in an impaired ability to assemble the Mn\(_{6}\)Ca cluster, or with an energetically unfavorable oxidation of manganese with or without an assembled Mn\(_{6}\)Ca cluster. Measurements of charge recombination in the presence of DCMU in whole cells of the D1-E333Q mutant (Fig. 2) show a dominant rapid kinetic phase with a \(t_{1/2}\) of 35 ms (45% of total). Measurements of the kinetics of charge recombination in whole cells of WT (TC35) and mutant D1-D170S show major components with \(t_{1/2}\) of 1.31 s (65%) and 15 ms (87%), respectively. It is thus likely that the differences in the rates of recombination between these different strains reflect differences in the ability of the oxidizing equivalent to be shared with a tertiary manganese. In the case of the WT this is predominantly the S1 state of the OEC, which is oxidized to S2. In the most extreme case of D1-D170S there is probably no tertiary electron donor manganese at all and the charge recombination occurs between Y\(_{Z}\) and Q\(_{A}\) through P\(_{680}\). That charge recombination between Q\(_{A}\) and P\(_{680}\) occurs with a \(t_{1/2}\) of 1 ms (32) implies an equilibrium constant of \(\sim 14\) for the reaction P\(_{680}\)\(^{-}\)Y\(_{Z}\) = P\(_{680}\)Y\(_{Z}\)(H\(^{+}\)). The case of D1-E333Q is intermediate between the two, with a slow phase (\(t_{1/2}\) = 1.82 s, 37%) likely reflecting that fraction of centers that has successfully assembled a functional Mn\(_{6}\)Ca complex (this work and Refs. 40 and 48) and that has advanced to the S2 state. The slight slowing of this component relative to that of WT may reflect either the influence of the mutation on the rate of S2Q\(_{A}\) recombination or some contribution from Q\(_{A}\)Mn\(^{3+}\) recombination in those centers that have not assembled the Mn\(_{6}\)Ca cluster, but which have been able to bind and oxidize Mn\(^{2+}\) during the lifetime of the charge separated state. The dominant fast phase (\(t_{1/2}\) = 35 ms, 45% of total) in the D1-E333Q mutants very likely arises from those centers that have been unable to assemble the Mn\(_{6}\)Ca cluster. This phase, two times slower than what is observed for charge recombination in D1-D170S, may reflect the influence of higher affinity binding of Mn\(^{2+}\) in D1-E333Q relative to D1-D170S on the reduction potential of Y\(_{Z}\)/E\(_{M}\) or of P680\(^{-}\)/P680.

Chu et al. (40) reported a sensitivity of the rate of charge recombination and of the rate of oxygen evolution to the intensity of light used to grow the cells in a number of D1-Glu\(^{333}\) mutants. We did not observe such sensitivity to light here (Table 1) as the rate of \(O_{2}\) evolution was consistently higher in D1-E333Q cells cultivated at 50 \(\mu\)E/m\(^{2}\)/s (29% of WT (TC35)) than at 2 \(\mu\)E/m\(^{2}\)/s (16% of WT (TC35)). The difference in \(O_{2}\) evolution rate was likely in large part due to a difference in the PSII center content in the cells at the two different light intensities (60 and 43% of WT, respectively). Furthermore, the rate of \(O_{2}\) evolution observed in the D1-E333Q cells grown in carboxys was close to that observed in dim light, implying that the core complexes isolated from such cells would not have been subject to light-induced damage. No differences were seen in \(O_{2}\) evolution rates or in PSII content per cell in WT (TC35 cells) cultivated at the same two light intensities and in carboxys. The differences observed here compared with Chu et al. (40) may reflect in part the fact that the mutations here were constructed in psbA3 and in Chu et al. (40) in psbA2. These two genes show some differences in light regulation of transcription (49).

Blockage of Charge Recombination by Mn\(^{2+}\)—Two sets of experiments were performed using PSII core complexes to examine the binding and oxidation of the first manganese of the OEC. Measurements of the ability of Mn\(^{2+}\) to block charge recombination between Q\(_{A}\) and the donor side indicate an increased \(K_{m,overall}\) in PSII core complexes isolated from both the D1-E333Q and the D1-E333H mutants (Table 1). The concentration of Mn\(^{2+}\) that blocks half of the centers is 0.3, 2.5, and 20 \(\mu\)M, respectively, for WT (TC31 and TC35), D1-E333Q, and D1-E333H. These are compared with \(K_{m,overall}\) of 1.3, 3.0, 3.4, and 1.7 \(\mu\)M for the D1-H332L, D1-D342V, D1-A344Stop, and D1-S345P mutant strains. The detailed fit of the concentration dependence to two hyperbolic binding components with dissociation constants \(K_{m1}\) and \(K_{m2}\) is shown in Table 1. The WT strains are clearly different from the mutant strains, with the latter showing a much less substantial tight binding component. Clearly the binding, although heterogeneous for all strains, is weaker for all of the C-terminal mutants as compared with WT. The replacement of D1-Glu\(^{333}\) with Gln, however, does not appear to be any more detrimental to the binding and oxidation of the first manganese than are mutations that affect other carboxylate residues, D1-D342V, D1-A344Stop, and D1-S345P (non-C-terminal processing strain (46)). These
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In a previous study, we found that D1-Glu<sup>333</sup> substitution reduces the first-order rate constant for the reduction of Y<sub>Z</sub> (<sup>1</sup>), which is consistent with the idea that this residue is involved in the oxidation of the first Mn<sup>2+</sup> binding site (Scheme 1). In the present study, we investigated the influence of the C-terminus of D1 on the first steps of OEC assembly.

**Influence of Mn<sup>2+</sup> on the Kinetics of Reduction of Tyrosine Y<sub>Z</sub>**—Measurements of the rates of reduction of Y<sub>Z</sub> (Fig. 6) indicate a difference between the PSII core complexes even prior to the addition of MnCl<sub>2</sub>. The first-order rate constants observed before MnCl<sub>2</sub> addition are 62 (73%), 45 (39%), and 1.4 s<sup>-1</sup> (80%) for the PSII core complexes isolated from the WT (TC35), D1-E333Q, and D1-D170S strains, respectively. An EDTA wash drops the rate in WT (TC31) to a value (4 s<sup>-1</sup>) similar to that observed in D1-D170S (1.4 s<sup>-1</sup>). Such adventitious Mn<sup>2+</sup> is likely due to trace contamination of the reagents during the preparation of the core complexes. The adventitious Mn<sup>2+</sup> is very tightly bound prior to the exciting flash. In the case of D1-D170S there is no pre-bound Mn<sup>2+</sup> and all of the Mn<sup>2+</sup> binds after the flash and very weakly. The adventitiously bound Mn<sup>2+</sup> is clearly able to influence the lifetime of Y<sub>Z</sub>; with the amount bound and/or its ability to donate to Y<sub>Z</sub> influenced by the nature of the mutation. The sensitivity of WT (TC31) to the EDTA wash and the ability to increase the percent contribution of the 45 s<sup>-1</sup> first-order rate constant upon addition of 1 μM MnCl<sub>2</sub> to the D1-E333Q core complexes does imply that the extent of occupancy of a binding site of varying affinity does differ between the different strains even before the addition of MnCl<sub>2</sub>.

In our earlier study, the very high affinity site (<i>K_m</i> < 1 μM) appeared in WT to account for little more than one-third of the Y<sub>Z</sub> relaxation in the absence of added MnCl<sub>2</sub>. In the present study (PSII core complexes from strain TC35), the percent occupancy of the high affinity site is higher (73%), although the second-order rate constant calculated using data from both TC31 and TC35 (2.6 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>) is similar and only slightly lower than in the earlier study (4 to 8.8 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>) (26) because of the manner in which the kinetics were fit. The Mn<sup>2+</sup> titrated in the second-order process for Y<sub>Z</sub> reduction binds subsequently to the actinic flash at a rate that increases with the Mn<sup>2+</sup> concentration, coming on and off many times prior to its oxidation. The earlier data were fit out to 1 s with two exponentials plus a constant, whereas the present data were fit out to 170 ms with a single exponential plus a constant. In the case of the D1-D170S mutant, the kinetic data measured earlier (26) were refit with a single exponential out to 1 s giving a second-order rate constant of 8.0 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>, only slightly larger than the 5.5 × 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup> measured earlier. This comparison places the second-order rate constant for the D1-D170S some 33–80-fold smaller than that of WT (TC31 and TC35). In the case of the D1-E333Q mutant, the rate constant observed for Y<sub>Z</sub> reduction in the absence of added MnCl<sub>2</sub> changes little upon the addition of 1 μM MnCl<sub>2</sub> (45 s<sup>-1</sup>) although its percent contribution increases. The pseudo first-order rate constant, however, increases to slightly less than that of WT upon increasing the Mn<sup>2+</sup> concentration to 10 μM, consistent with the idea that a very high affinity binding component is affected by this and other C-terminal mutations.

**Modeling of Early Manganese Coordination**—The site-directed mutations, described in this and in previous work, that have the most impact on the <i>K_m</i> for binding and oxidation of Mn<sup>2+</sup> in Mn-depleted core complexes are D1-D170S, Ala, and Asn. D1-D170E and Cys are the most conservative mutations with the least impact on <i>K_m</i>. The presence of the carboxylate group or at least an anionic charge at this site, therefore appears to contribute to the Mn<sup>2+</sup> affinity. The consequence of the replacement of D1-Glu<sup>333</sup> with Gln or His is much smaller, with the former quite similar to D1-D342V, D1-A344Stop, and D1-S345P. The C-terminal mutations therefore fall into a second class of weaker effects on first Mn<sup>2+</sup> binding and oxidation. The simplest model to explain these observations is one in which D1-Asp<sup>170</sup> is a key component of the high affinity Mn<sup>2+</sup> binding site. The C-terminal residues mentioned above all contribute, but more weakly (D1-His<sup>332</sup> most weakly), to the high affinity binding site, most likely through their contributions of anionic charges that increase the local concentration of Mn<sup>2+</sup> available to the high affinity binding site at D1-Asp<sup>170</sup> (Scheme 1). That all of these mutations influence the binding behavior of the first Mn<sup>2+</sup> indicates that they are near neighbors in the three-dimensional environment of D1-Asp<sup>170</sup> despite their distance in the primary sequence of D1. The difference in behavior of D1-E333Q and His could mean that the latter residue is particularly close to D1-Asp<sup>170</sup> inhibiting sterically or electrostatically the binding process.

One could argue that the C-terminal mutations have some influence on the conformation of the high affinity binding site, a view somewhat different from the electrostatic model described above. However, both an electrostatic model and a conformational one imply that the C terminus of the D1-polypeptide assumes a conformation that enhances the high affinity binding of the first manganese to be assembled into the OEC. This conformation must place the C-terminal region in the vicinity of D1-Asp<sup>170</sup> at the very beginning of the binding process in a conformation close to that observed in the three-
dimensional x-ray structures where the Mn₄Ca cluster is fully assembled.

Recent x-ray absorption spectroscopy studies have shown that, under conditions of x-ray diffraction data collection on PSII crystals, the production of solvated electrons significantly reduces the manganese complex, largely to the Mn(II) state (29, 30). Consequently, reservations have been expressed regarding the accuracy of the described coordination environments of the component manganese and calcium of the OEC in the x-ray crystal structures (9, 29, 30). The coordination environments as depicted in the x-ray structures may therefore be somewhat different from those that actually exist in the more oxidized dark stable S0 and S1 states of the OEC. The 3-Å resolution of the best x-ray structure still allows for some uncertainty in the ligand assignment. These uncertainties in the precise location of the C-terminal manganese-coordinating residues do not affect the conclusions arrived at in this paper that only require a close proximity of these residues to D1-Asp170 prior to or during coordination of the first bound manganese by D1-Asp170 and D1-Glu333 and may even argue against it, they do not exclude such co-coordination from existing in the fully assembled Mn₄Ca cluster as depicted in the 3-Å crystal structure (5).

Acknowledgment—We very gratefully acknowledge insightful comments from Dr. Richard J. Debus.

REFERENCES

1. Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) *Nature* 409, 739–743
2. Kamiya, N., and Shen, J. R. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 98–103
3. Biesiadka, J., Loll, B., Kern, J., Irrgang, K. D., and Zouni, A. (2004) *Phys. Chem. Chem. Phys.* 6, 4733–4736
4. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Paddock, M. L., Feher, G., and Okamura, M. Y. (2006) *Biochemistry* 45, 134–138
5. Diner, B. A., and Britt, R. D. (2005) in *Photosystem II: The Light-driven Water:Plastoquinone Oxidoreductase* (Wydrzynski, T. J., and Satoh, K., eds) pp. 207–233, Springer, Berlin
6. Diner, B. A., and Britt, R. D. (2005) in *Photosynthesis: The Light Reactions* (Orr, D. R., and Yocum C. F., eds) pp. 213–247, Kluwer Academic Publishers, Dordrecht
7. Yachandra, V. K. (2005) in *Photosystem II: The Light-driven Water:Plastoquinone Oxidoreductase* (Wydrzynski, T. J., and Satoh, K., eds) pp. 235–260, Springer, Berlin
8. Nuijs, A. M., Van Gorkom, H. J., Plijter, J. J., and Duyens, L. N. M. (1986) *Biochim. Biophys. Acta.* 848, 167–175
9. Petrouleas, V., and Crofts, A. R. (2005) in *Photosystem II: The Light-driven Water:Plastoquinone Oxidoreductase* (Wydrzynski, T. J., and Satoh, K., eds) pp. 177–206, Springer, Berlin
10. Haumann, M., Mueller, C., Liebisch, P., Juzzolino, L., Dittmer, J., Grabolle, M., Neisius, T., Meyer-Klaucke, W., and Dau, H. (2005) *Biochemistry* 44, 1894–1908
11. Diner, B. A. (2001) *Biochim. Biophys. Acta.* 1503, 147–163
12. Debus, R. J. (2001) *Biochim. Biophys. Acta.* 1503, 164–186
13. Debus, R. J. (2005) in *Photosystem II: The Light-driven Water:Plastoquinone Oxidoreductase* (Wydrzynski, T. J., and Satoh, K., eds) pp. 261–284, Springer, Berlin
14. Ananyev, G. M., Sakiyan, I., Diner, B. A., and Dismukes, G. C. (2002) *Biochemistry* 41, 974–980
15. Nixon, P. J., and Diner, B. A. (1992) *Biochemistry* 31, 942–948
16. Diner, B. A., and Nixon, P. J. (1992) *Biochim. Biophys. Acta.* 1101, 134–138
17. Boerner, R. J., Nguyen, A. P., Barry, B. A., and Debos, R. J. (1992) *Biochemistry* 31, 660–6672
18. Campbell, K. A., Force, D. A., Nixon, P. J., Dole, F., Diner, B. A., and Britt, R. D. (2000) *J. Am. Chem. Soc.* 122, 3759–3761
19. Yano, J., Kern, J., Irrgang, K. D., Lattimer, M. J., Bergmann, U., Glatzel, P., Pushkar, Y., Biesiadka, J., Loll, B., Sauer, K., Messinger, J., Zouni, A., and Yachandra, V. K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 12047–12052
20. Grabolle, M., Haumann, M., Mueller, C., Liebisch, P., and Dau, H. (2006) *J. Biol. Chem.* 281, 4580–4588
21. Williams, J. G. K. (1988) *Methods Enzymol.* 167, 766–778
22. Metz, J. G., Nixon, P. J., Rognier, M., Brudvig, G. W., and Diner, B. A. (1989) *Biochemistry* 28, 6960–6969
23. Lichtenthaler, H. K. (1987) *Methods Enzymol.* 148, 350–382
24. Tang, X. S., and Diner, B. A. (1994) *Biochemistry* 33, 4594–4603
25. Rognier, M., Nixon, P. J., and Diner, B. A. (1990) *J. Biol. Chem.* 265, 6189–6196
26. Diner, B. A., and Britt, R. D. (2005) in *Advances in Photosynthesis* (Sybesma, C., ed) Vol. I, pp. 477–480, Martinus Nijhoff/Dr. W. Junk, The Hague
27. Graige, M. S., Paddock, M., Bruce, J. M., Feher, G., and Okamura, M. Y. (1996) *J. Am. Chem. Soc.* 118, 9005–9016
28. Mauzerall, D. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 1358–1362
29. Johnson, G. N., Rutherford, A. W., and Krieger, A. (1995) *Biochim. Biophys. Acta.* 1229, 202–207
30. Robinson, H. H., and Crofts, A. R. (1984) in *Advances in Photosynthesis Research* (Sybesma, C., ed) Vol. I, pp. 477–480, Martinus Nijhoff/Dr. W. Junk, The Hague
31. Mohamed, A., and Jansson, C. (1989) *Plant Mol. Biol.* 13, 693–700

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VOLUME 282 • NUMBER 10 • MARCH 9, 2007