Blocking of Electron Donation by Mn(II) to $Y_Z^\bullet$ following Incubation of Mn-Depleted Photosystem II Membranes with Fe(II) in the Light[†]

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ABSTRACT: The donation of electrons by Mn(II) and Fe(II) to $Y_Z^\bullet$ through the high-affinity (HA_Z) site in Mn-depleted photosystem II (PSII) membranes has been studied by flash-probe fluorescence yield measurements. Mn(II) and Fe(II) donate electrons to $Y_Z^\bullet$ with about the same efficiency, saturating this reaction at the same concentration (ca. 5 μM). However, following a short incubation of the membranes with 5 μM Fe(II), but not with Mn(II) in room light, added Mn(II) or Fe(II) can no longer be photooxidized by $Y_Z^\bullet$. This blocking effect is caused by specifically bound, photooxidized Fe [≥Fe(III)] and is accompanied by a delay in the fluorescence yield decay kinetics attributed to the slowing down of the charge recombination rate between Q_a^- and $Y_Z^\bullet$. Exogenously added Fe(III), on the other hand, does not donate electrons to $Y_Z^\bullet$, does not block the donation of electrons by added Mn(II) and Fe(II), and does not change the kinetics of the decay of the fluorescence yield. These results demonstrate that the light-dependent oxidation of Fe(II) by $Y_Z^\bullet$ creates an Fe species that binds at the HA_Z site and causes the blocking effect. The pH dependence of Mn(II) electron donation to $Y_Z^\bullet$ via the HA_Z site and of the Fe-blocking effect is different. These results, together with sequence homologies between the C-terminal ends of the D1 and D2 polypeptides of the PSII reaction center and several diiron−oxo enzymes, suggest the involvement of two or perhaps more (to an upper limit of four to five) bound iron cations per reaction center of PSII in the blocking effect. Similarities in the interaction of Fe(II) and Mn(II) with the HA_Z Mn site of PSII during the initial steps of the photoactivation process are discussed. The Fe-blocking effect was also used to investigate the relationship between the HA_Z Mn site and the HA sites on PSII for diphenylcarbazide (DPC) and NH_2OH oxidation. Blocking of the HA_Z site with specifically bound Fe leads to the total inhibition of electron donation to $Y_Z^\bullet$ by DPC. Since DPC and Mn(II) donation to PSII is noncompetitive [Preston, C., and Seibert, M. (1991) *Biochemistry 30,* 9615−9624], the Fe bound to the HA_Z site can also block the DPC donation site. On the other hand, electron donation by NH_2OH to PSII still occurs in Fe-blocked membranes. Since hydroxylamine does not reduce the Fe [≥Fe(III)] specifically bound to the HA_Z site, NH_2OH must donate to $Y_Z^\bullet$ through its own site or directly to $P680^+$.

Oxygenic photosynthetic organisms oxidize water using a light-active pigment/protein complex called photosystem II (PSII)[1] that contains the oxygen-evolving complex (OEC). The OEC includes the tetranuclear manganese cluster, a calcium cation, and chloride anion(s) (for reviews see refs *1*−*6*). Recently, a metalloradical mechanistic model of the OEC has been proposed, in which the redox-active tyrosine Y_Z, located at position 161 on the D1 PSII reaction center (RC) polypeptide (*Synechocystis* notation), is also included (*7*−*9*). Situated at a distance of 6−11.5 Å from the Mn cluster (*10*−*12*), the neutral tyrosine radical $Y_Z^\bullet$ is formed after the oxidation of Y_Z by $P680^+$, the oxidized primary electron donor, on a nanosecond time scale [15−300 ns (*13*)]. The tyrosine participates not only as a redox link between $P680^+$ and the Mn cluster but also as a proton (*7*) or hydrogen (*8*) abstraction agent from substrate water molecules bound to the Mn cluster.

The Mn cluster/Ca cation complex can be extracted from the OEC with Tris, hydroxylamine, or a lipophilic chelator (*14*) without significant inactivation of the cation binding sites, as demonstrated by subsequent reactivation of the apoenzyme (a process called photoactivation) with the re-addition of Mn and Ca. In fact, Mn-depleted PSII membranes

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[1] Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; F_0, fluorescence emitted by a sample at low light levels prior to flash excitation; $(F - F_0)/F_0$, fluorescence yield; F_{fin}, final fluorescence yield detected after decay of the flash-induced F_{max}; F_{max}, maximum fluorescence yield following actinic flash excitation; HA_Z, high-affinity electron donation site to $Y_Z^\bullet$ by exogenous donors; LA, low-affinity electron donation site by exogenous donors; K_d, dissociation constant of the enzyme−ligand complex; K_m, Michaelis−Menten constant for an enzymatic reaction; MES, 2-(*N*-morpholino)-ethanesulfonic acid; OEC, oxygen-evolving complex; P680, primary electron donor in PSII; PSII, photosystem II; Q_a, primary quinone acceptor of PSII; Q_b, secondary quinone acceptor of PSII; RC, reaction center; Tris, tris(hydroxymethyl)aminomethane; Y_Z (D1-Tyr161), redox-active tyrosine, the first electron donor to $P680^+$ in PSII; Y_D, redox-active tyrosine (D2-Tyr160), an alternative electron donor to $P680^+$ in PSII.

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have been used extensively for the investigation of possible Mn donation sites. According to kinetic studies of the photoactivation process, the initial stage of reassembly of the Mn cluster involves the binding of Mn(II) to the apoenzyme and its photooxidation by $Y_Z^{\bullet}$. The formation of this unstable mononuclear Mn(III) intermediate is followed by the rapid binding of a second Mn(II) and formation of an unstable binuclear intermediate, Mn(III)−Mn(II). This intermediate rearranges in the dark with a 150 ms half-time, which allows the photooxidation of the second Mn(II) to occur, forming a stable Mn(III)−Mn(III) dimer (*14*−*19*). Ligation of two other Mn cations in the dark completes the formation of the active, tetranuclear Mn cluster.

Under nonphotoactivating conditions, addition of Mn(II) to Mn-depleted membranes results in the coordination of one manganese cation to a high-affinity site and its oxidation by light, as has been shown by atomic absorption (*20*, *21*) and EPR (*21*, *22*) spectroscopies. The binding to and oxidation of Mn(II) in Mn-depleted PSII membranes is believed to occur at the native Mn-binding site of the OEC. Therefore, the process of oxidation of added Mn(II) to Mn-depleted PSII membranes has been investigated with the goal of identifying the site that binds at least the initial Mn in the assembly of the OEC (*20*−*38*). Electron donation by added Mn(II) has been analyzed by the application of a number of spectroscopic methods such as monitoring the reduction of dichlorophenolindophenol (DCIP), a PSII electron acceptor (*23*, *25*, *28*−*32*, *35*); measuring the yield and decay of flash-induced chlorophyll *a* fluorescence (*24*, *32*−*34*, *39*, *40*); and observing $Y_Z^{\bullet}$ reduction by EPR (*21*, *26*, *30*). Several indirect methods of investigating Mn-binding sites have also been used, including inhibition of diphenylcarbazide (DPC) oxidation by micromolar concentrations of Mn(II) (the DPC-inhibition assay) (*28*, *31*, *33*−*39*), the effect of metal cations on the reduction of $P680^+$ (*30*, *41*, *42*), and the application of amino acid modifiers (*20*, *30*, *31*, *33*, *34*, *38*).

Steady-state, light-induced DCIP reduction with Mn(II) as the electron donor has identified a high-affinity donation site with $K_m \leq 1$ μM (*24*, *28*, *30*, *33*, *34*) and a low-affinity site with $K_m \sim 100-200$ μM (*23*, *28*, *29*). Electron donation by Mn(II) at micromolar concentrations, stimulated by H_2O_2 or other reductants such as NH_2OH or NH_2NH_2, also occurs through the high-affinity donation site with a K_m value of <1 μM (*25*, *35*). The reduction of $Y_Z^{\bullet}$ by Mn(II) takes place with the same Michaelis−Menten constant value of about 1 μM (*27*). These data indicate that the high-affinity site for Mn(II) reflects the donation of electrons by Mn(II) to $Y_Z^{\bullet}$, and we will call it the HA_Z Mn site. Certain measurements have attributed a $K_m \sim 10-15$ μM to the same site (*23*, *29*, *30*, *33*, *34*). It is believed that this apparent higher K_m is a manifestation of the experimental procedures applied and does not reflect the actual higher affinity for Mn(II) electron donation to $Y_Z^{\bullet}$ (*23*, *35*). The nature of the low-affinity (LA) donation site with $K_m > 100$ μM is not clear, although $Y_D^{\bullet}$ has been suggested as a possible oxidant for exogenous donors (*23*). Two sites of oxidation with either high or low affinity have also been found for DPC and I^- (*23*). The high-affinity DPC site [K_m around 40 μM (*23*, *43*)] most likely reflects electron donation to $Y_Z^{\bullet}$, while the nature of the low-affinity site [K_m around 1−2 mM (*23*, *43*)] is not clear yet [although again $Y_D^{\bullet}$ has been proposed as the oxidant (*23*)].

The interaction of other metal cations, including Ca^{2+} and Mg^{2+} (*36*, *38*), Co(II) and Zn(II) (*36*, *38*, *39*), Ba(II) and Cu(II) (*38*), Fe(II) and Fe(III) (*44*, *45*), and Cs^+ (*46*), with the HA_Z site has also been investigated. With the exception of Fe(II) (*44*) and Cs^+ (*46*), the efficiency of interaction of all these cations with the HA_Z site was significantly less than that of Mn(II). Similarities in the interactions of Fe(II) and Mn(II) with HA_Z in the presence of DPC were described previously (*44*, *45*). The midpoint redox potential, E_m, of the Fe(II)/Fe(III) couple is +0.77 V or less in the presence of various binding groups [for example, it is about −0.3 V in the presence of a pyrophosphate group (*47*)]. This E_m range is appropriate for Fe(II) to function as an electron donor to Mn-depleted PSII membranes. Indeed, light-dependent oxidation of Fe(II) during incubation with Tris-washed PSII membranes has been demonstrated (*44*). Involvement of other oxidizing components [possibly $Y_D^{\bullet}$ (*23*)] in the oxidation of Fe(II) on the donor side of Mn-depleted PSII membranes is possible, as has been shown for Mn(II) and DPC (*23*, *28*, *39*). Significantly, amino acid sequence analyses of the D1 and D2 proteins demonstrated the presence of conservative motifs and residues on the C-termini of D1 and D2 that are known to coordinate the diiron cluster in diiron−oxo enzymes such as ribonucleotide reductase and methane monooxygenase [enzymes in which Mn can be substituted structurally but not functionally for the iron (*48*, *49*)]. As a consequence, one of us suggested that these newly recognized motifs might help to coordinate the dimanganese cluster in PSII (*50*).

This paper compares the characteristics of electron donation to $Y_Z^{\bullet}$ by Mn(II) and Fe(II) cations using flash-probe fluorescence yield decay methods. We found that light-dependent oxidation of Fe(II) by Mn-depleted PSII membranes is accompanied by the irreversible binding of photooxidized Fe to the HA_Z Mn site, and this "Fe block" prevents further oxidation of both Mn(II) and Fe(II) by $Y_Z^{\bullet}$. Similarities between the initial process of photoactivation and the irreversible binding of the photooxidized Fe, possibly as an Fe(III)−Fe(III) cluster, will be discussed. We have also used the Fe-blocking effect to investigate the relationship between the HA_Z site and the donation sites to $Y_Z^{\bullet}$ for other exogenous electron donors such as DPC and NH_2OH.

MATERIALS AND METHODS

PSII-enriched membrane fragments (BBY type) were prepared from market spinach (*51*) and resuspended in buffer A (50 mM MES/NaOH buffer, pH 6.5, 15 mM NaCl, and 0.4 M sucrose). Chlorophyll concentrations and Chl *a*/*b* ratios were determined in 80% acetone, according to the method of Porra et al. (*52*), O_2 evolution rates were 400−550 μmol·(mg of Chl^{-1})·h^{-1}, and the membranes were stored in liquid nitrogen until use. Manganese depletion was accomplished by incubating thawed PSII membranes (0.5 mg of Chl/mL) in 1 M Tris-HCl buffer, pH 9.4, containing 0.4 M sucrose for 30 min at 5 °C in room light (*38*).

Concentrated stocks of 0.5 mM $MnCl_2$ and 0.5 mM $FeSO_4$ in doubly distilled water were made up just prior to the experiments. Little oxidation of Fe(II) in the stock solution (pH 5.6) was observed after 5 h at room temperature under room light. In contrast, the stability of Fe(II) decreased in buffer A (pH 6.5), but only about 5% of the Fe(II) was

oxidized after a 1 h incubation. Oxidation of Fe(II) in the buffer during the 5 min light incubation period required by our experimental protocol was insignificant (see the next paragraph for the procedure used to check for Fe stability). Formation of ferrous hydroxide does not occur at the concentrations and pHs used (stock solution, pH 5.6, 0.5 mM $FeSO_4$; buffer A, pH 6.5, $\leq$100 μM $FeSO_4$) (*53*). Stock solutions of $FeCl_3$ (1 mM) were prepared as a soluble and stable complex of ferric iron with sucrose as described by Charley et al. (*54*).

To determine the pH dependence of electron donation by Mn(II) to $Y_Z^{\bullet}$, Mn-depleted membranes were resuspended in MES buffer (50 mM MES, 0.4 M sucrose, 15 mM NaCl) at pH <7.0 or in Tricine buffer (50 mM Tricine, 0.4 M sucrose, 15 mM NaCl) at pH >7.0. No differences in the electron donation rates by Mn(II) in MES and Tricine buffers at the same pH were found. The pH dependence of the iron-blocking effect was measured in MES/NaOH buffer at all pHs because Tricine can extract iron cations bound to the HA_Z site. During measurements of the pH dependence of the blocking effect, the pHs of the buffers at pH >7.0 were checked directly after addition of all components to the sample. The experiments were started by incubating Mn-depleted PSII membranes (37.5 μg of Chl/mL) in buffer A at the indicated pHs for 2 min with 15 μM Fe(II) under room light. The samples were then centrifuged, and the pellets were resuspended in buffer A at pH 6.5. The presence of iron cations blocking the HA_Z site was determined by measurement of electron donation by 10 μM Mn(II) (see below). The stability of Fe(II) in buffer at pHs >6.5 was determined with *o*-phenanthroline by the formation of the red-colored ferrous phenanthrolinate complex. The amount of this complex was determined spectrophotometrically at $\lambda = 512$ nm (*55*).

Steady-state, light-induced DCIP photoreduction was measured at 600 nm using a computer-controlled Aminco DW2A spectrophotometer in the split-beam mode as described (*39*). Initial rates were calculated from measurements of initial slopes of the kinetics obtained within 3 s of sample illumination. The assay medium contained 5 μg of Chl/mL of PSII, 40 μM DCIP, and added donors in buffer A. Each DCIP photoreduction rate reported was an average of three to four experiments. Oxygen evolution by PSII samples was measured polarographically with a Clark electrode in the presence of the artificial electron acceptor 2,6-dichloro-*p*-benzoquinone (Eastman Kodak Co., Rochester, NY) purified by recrystallization (*56*).

The decay of the flash-induced variable fluorescence yield was measured at room temperature using a home-built, flash-probe instrument capable of 100 μs time resolution (*39*). Membrane suspensions were excited with saturating actinic, single-turnover flashes (3 μs) provided by an EG&G FX201 xenon flash lamp filtered through a Corion LS-650 low band-pass filter. Weak monitoring flashes were provided by an array of Hewlett-Packard HLMA-CHOO light-emitting diodes (LED, 621 nm emission, 100 Hz flash rate). Variable fluorescence yields were monitored at $\geq$715 nm as a function of time. Samples for fluorescence measurements were dark adapted and contained 25 μg of Chl/mL (ca. 0.12 μM PSII centers) and 37.5 μM DCMU in buffer A. Data analysis used Data Translation Global Lab software and a DT2839 A/D board mounted in an ALR 486 PC (*39*).

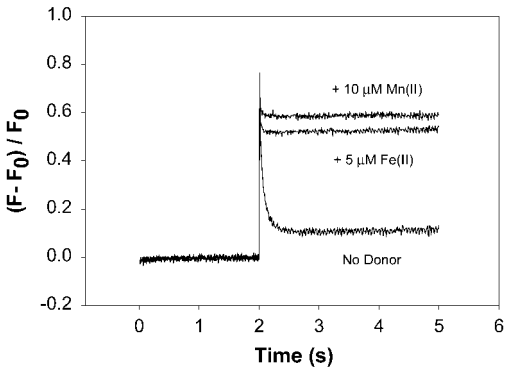

FIGURE 1: Effect of Mn(II) and Fe(II) on the decay of the flash-probe chlorophyll fluorescence yield. Tris-washed (Mn-depleted) PSII membrane fragments at 25 μg of Chl/mL in buffer A (pH 6.5) were illuminated with a single-turnover flash in the absence or presence of either 10 μM $MnCl_2$ or 5 μM $FeSO_4$. Each sample contained 37.5 μM DCMU. The samples were dark adapted before measurement. Three measurements were averaged. See text for other experimental conditions. $(F - F_0)/F_0$ is the fluorescence emission normalized to F_0, the fluorescence emitted by the samples prior to flash excitation.

RESULTS

In Mn-depleted PSII membranes, a single-turnover saturating flash results in charge separation across the RC complex and subsequent generation of the high-fluorescence state, $Y_Z^{\bullet}Q_a^-$. The fluorescence yield [$(F - F_0)/F_0$, where F_0 is the fluorescence emitted by the sample prior to flash excitation] at each time point following the flash is determined by the population of Q_a in the reduced state (Q_a^-). Oxidation of Q_a^- following the flash is accompanied by a decrease in the fluorescence yield from F_{max} to a low steady-state level F_{fin}. F_{max} and F_{fin} represent the amount of Q_a^- accumulated respectively at 10 ms and 3−5 s after the flash. If the normal path of Q_a^- oxidation via Q_b is interrupted by DCMU, Q_a^- is oxidized by $Y_Z^{\bullet}$ with a recombination time of 15−100 ms (*39*, *40*, *57*−*59*). Addition of exogenous electron donors to Mn-depleted, DCMU-treated PSII membranes results in a faster reduction of $Y_Z^{\bullet}$ by the added donors, and in an increase in F_{fin}, due to the lack of oxidation of Q_a^- by $Y_Z^{\bullet}$. Thus, in the presence of DCMU (which blocks Q_a to Q_b electron transport), the value of the nondecaying fluorescence yield, F_{fin}, reflects the affinity of exogenous donors, and after appropriate normalization, the fluorescence data can be used to determine the kinetic parameters of electron donation to the membranes (*21*, *33*, *34*). We used the ratio F_{fin}/F_{max} to represent the fraction of nonrecombined Q_a^- at the end of the measurement and the ratio $(F_{max} - F_{fin})/F_{max}$ to represent the fraction of recombined Q_a^- at the same time point. In the presence of an exogenous donor and DCMU, the ratio F_{fin}/F_{max} will be larger than in the absence of the exogenous reagents due to a lower amount of recombined Q_a^-. The value of the F_{fin}/F_{max} ratio will also depend on the concentration and efficiency of the donor. Therefore, changes in the F_{fin}/F_{max} ratio upon addition of donors were used in this study to measure electron donation to PSII.

Figure 1 shows typical traces of the changes in the flash-induced fluorescence yield measured in the absence or presence of the electron donors, $MnCl_2$ and $FeSO_4$. The addition of either Fe(II) or Mn(II) leads to an increase in the F_{fin} level, demonstrating that both cations can donate

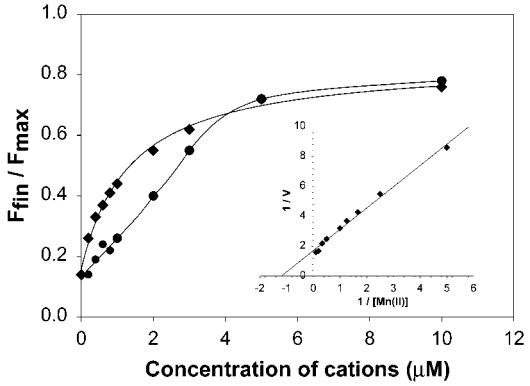

FIGURE 2: F_{fin}/F_{max} ratio as a function of added Mn(II) (◆) or Fe(II) (●) cations. F_{fin}/F_{max} is a measure of the number of PSII reaction centers that do not undergo $Y_Z^{\bullet}Q_a^-$ recombination after light-induced charge separation due to the reduction of $Y_Z^{\bullet}$ by an exogenous electron donor. See Figure 1 legend and the text for other experimental details. Insert: Lineweaver−Burk plot of the above data. Values for V were estimated as $[(F_{max} - F_{fin})/F_{max}^{\text{without donor}} - (F_{max} - F_{fin})/F_{max}^{\text{with donor}}]$.

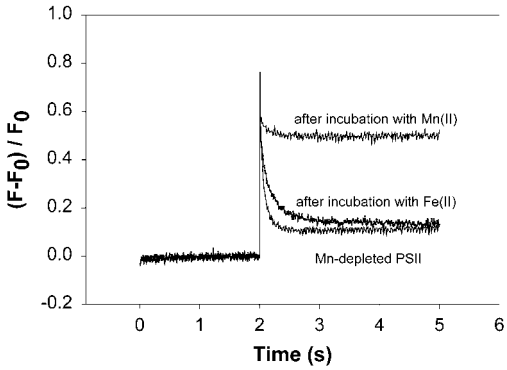

FIGURE 3: Decay of the flash probe fluorescence yield in Mn-depleted PSII membranes preincubated with 5 μM Mn(II) or Fe(II) for 5 min under room light. Control membranes were not preexposed to light. The concentration of Mn-depleted PSII membranes was 25 μg of Chl/mL. After incubation, the samples were dark adapted for ≥15 min. DCMU (37.5 μM) was added to the sample before the fluorescence measurement.

electrons to $Y_Z^{\bullet}$. Figure 2 shows that the level of F_{fin} increases with the concentration of added Mn(II) or Fe(II) and reaches F_{max} at concentrations somewhat higher than 5 μM for both donors. This suggests about the same donation efficiency for Mn(II) and Fe(II) cations at the high-affinity site. However, the dependence of the F_{fin}/F_{max} ratio on cation concentration is different for Mn(II) and Fe(II) at lower concentrations. In the case of Fe(II), the sigmoidal relationship between F_{fin}/F_{max} and the concentration of added iron reflects cooperativity in the interaction of these cations with the donor site. Control experiments indicated that the sigmoidicity was not the result of an artifact related to a larger fractional loss of Fe(II) at the lower Fe(II) concentrations due to dark reactions with dissolved O_2 [only 1% of 5 μM Fe(II) in buffer A is oxidized in 5 min, and the calibration curve for added Fe(II) is linear between 0 and 1.5 μM Fe(II) using the *o*-phenanthroline assay] or with endogenous oxidants [only 1.5% of 20 μM Fe(II) is oxidized in buffer A containing Mn-depleted PSII membranes after a 20 min incubation] prior to the flash. Although we cannot fully exclude the possibility that small amounts of Fe(II) are oxidized by endogenous oxidants at low Fe(II) concentrations, the data in Figure 2 strongly suggest that the efficiency of electron donation by Fe(II) to $Y_Z^{\bullet}$ depends on the concerted binding of two or more cations.

We analyzed the Mn(II) data from Figure 2 as a Lineweaver−Burke plot, where $1/V$ [$V = (F_{max} - F_{fin})/F_{max}^{\text{without donor}} - (F_{max} - F_{fin})/F_{max}^{\text{with donor}}$] was plotted as a function of 1/[Mn(II)] (Figure 2 insert). This allowed us to determine a K_m value for Mn(II) electron donation to $Y_Z^{\bullet}$. The K_m value (0.83 μM) obtained is consistent with the values of the dissociation constant for Mn(II) [$K_d < 1$ μM (*33*, *34*) and 1.3 μM (*21*)] determined by similar methods. Experimental anomalies associated with the shape of the Fe(II) titration curve (Figure 2) have prevented us from determining a specific K_m for Fe(II) donation.

It is known from the DPC-inhibition assay that micromolar concentrations of added Mn(II) (*20*, *28*, *31*−*39*) and Fe(II) (*44*, *45*) inhibit the donation of electrons by DPC to $Y_Z^{\bullet}$ in Mn-depleted PSII membranes, which is reflected in a 50% decrease in the rate of DCIP reduction. The inhibition is due to binding of Mn(II) to the high-affinity site with $K_d < 1$ μM (*32*−*39*). The remaining 50% (the noninhibited rate) is believed to be due to electron donation by DPC to $Y_D^{\bullet}$ (*23*, *39*). The inhibition of DPC photooxidation by Mn(II) binding to the high-affinity site can be reversed easily by washing the membranes with buffer devoid of Mn. Recovery of 100% of the initial DPC-oxidation activity was reported by application of this procedure to samples that had been preincubated with 5 μM $MnCl_2$ under saturating light for several seconds (*45*). The inhibition of electron transport from DPC to DCIP by Fe(II), on the other hand, becomes irreversible if Mn-depleted PSII membranes are incubated with Fe(II) under room light for as little as 5 min (*45*). Such preparations still show the same noninhibited 50% rate of DPC-supported DCIP reduction (through the LA site) after removal of unreacted iron cations by centrifugation (*45*) but no donation from DPC to $Y_Z^{\bullet}$. Taking this effect into account, we studied the HA_Z site in preparations that had been incubated for short times (5 min) with 5 μM Fe(II) or 5 μM Mn(II) cations under room illumination in the *absence* of DPC.

Figure 3 shows the flash-induced fluorescence decay traces of control Mn-depleted PSII membranes and similar membranes after a 5 min preincubation with either Fe(II) or Mn(II) cations under room light (about 4 $\mu E \cdot m^{-2} \cdot s^{-1}$; the control was not preincubated in the light in this case). We observed two effects of the Fe(II) treatment: (i) the donation of electrons to $Y_Z^{\bullet}$ was absent, although nonoxidized Fe(II) was still present in the buffer after incubation (determined as in Materials and Methods but not shown), and (ii) the decay of the fluorescence yield kinetics was significantly slower (compare to the control trace). On the other hand, preincubation of PSII membranes with Mn(II) did not inhibit electron transfer from Mn(II) to $Y_Z^{\bullet}$, and fast reduction of $Y_Z^{\bullet}$ by the Mn(II) was still observed after the treatment.

The lack of electron donation to $Y_Z^{\bullet}$ in Fe(II)-treated membranes was not due to the absence of Fe(II) cations in the buffer, since the addition of 10 or 100 μM Fe(II) before the measurement had little additional effect on the fluorescence decay (data not shown). Moreover, added Fe(II) cations were still not able to donate electrons even after extensive washing of the Fe(II)-treated membranes (Table 1, compare row 5 to rows 1 and 3), demonstrating irreversible binding

Table 1: Donation of Electrons by Fe(II) and Mn(II) to $Y_Z^{\bullet}$ in Mn-Depleted PSII [PSII(−Mn)] Preparations after the Indicated Treatments

sample and treatment	donation (relative units)[a] +Mn(II),[b] 10 μM	+Fe(II),[b] 10 μM
1. PSII(−Mn), control	0.63	0.63
2. PSII(−Mn) after 5 min incubation under room light[c]	0.38	0.52
3. PSII(−Mn) after 5 min incubation under room light with 5 μM Fe(II)	0.03	0.03
4. PSII(−Mn) after 5 min incubation in the dark with 5 μM Fe(II)	0.72	0.68
5. PSII(−Mn) after 5 min incubation under room light with 5 μM Fe(II), followed by centrifugation and resuspension in buffer A	0.08	0.07
6. PSII(−Mn) with 10 μM Fe(III)	0.7	0.68
7. PSII(−Mn) after 5 min incubation under room light with 10 μM Fe(III)[d]	0.41	0.54
8. PSII(−Mn) after 5 min incubation under room light with 5 μM Fe(II) (HA_Z site blocked); then sample was incubated for 2 min in Tricine buffer,[e] followed by centrifugation and resuspension in buffer A	0.48[f]	nd[g]

[a] Donation level is determined from the equation $(F_{max} - F_{fin})/F_{max}^{without\ donor} - (F_{max} - F_{fin})/F_{max}^{with\ donor}$ (see text). [b] Mn(II) or Fe(II) was added after the indicated treatment. [c] Light incubation can damage PSII(−Mn) material in the absence of electron donors. While this experiment indicates that 5 min of illumination under our conditions can do some damage, most of the Mn(II) and Fe(II) donation activity still remains. [d] Note that there is a reducing side effect of exogenous Fe(III) treatment at higher Fe(III) concentrations in the light. Fe(III) can accept electrons from the acceptor side of PSII membranes in the absence of DCMU under these conditions. [e] Tricine buffer was buffer A containing 50 mM Tricine (pH 8.0) instead of 50 mM MES. MES buffer at pH 8.0 did not extract bound iron, but at pH 8.4 some extraction was starting to be observed. [f] Concentration of Mn(II) was 5 μM. [g] Not determined.

Table 2: Electron Transport from Mn(II) to DCIP in Mn-Depleted PSII with a "Blocked" HA_Z Site

Mn-depleted PSII membranes	rate of DCIP photoreduction [μmol of $DCIPH_2$·(mg of Chl^{-1})·h^{-1}]
without additions	12 ± 1[a]
+10 μM Mn(II)	28 ± 3, $\Delta = 16$[b]
after incubation with Fe(II)[c]	16 ± 3
after incubation with Fe(II), then 10 μM Mn(II) was added	20 ± 1, $\Delta = 4$
after incubation with Mn(II)[c]	22 ± 1
after incubation with Mn(II), then 10 μM Mn(II) was added	37 ± 3, $\Delta = 15$

[a] The results of two experiments with three to five measurements each were averaged. [b] The symbol Δ is a difference between the rate of DCIP photoreduction in the presence and absence of added Mn(II). [c] Mn-depleted PSII membranes (25 μg of Chl/mL) were incubated with 5 μM $FeSO_4$ or 5 μM $MnCl_2$ for 5 min under room light in the absence of DCMU, and then the rate of Mn(II) photooxidation was measured using DCIP as an acceptor.

of iron cations to the donation site under these conditions [however, iron cations blocking the HA_Z site can be extracted by Tricine treatment at alkaline pH (Table 1, row 8), and this regenerates the ability of Mn(II) of Fe(II) to donate to PSII]. Blocking of the HA_Z donation site with bound iron cations prevents donation of electrons to $Y_Z^{\bullet}$ not only by Fe(II) but also by Mn(II) (see also Table 1, rows 3 and 5). This suggests that the sites for electron donation to $Y_Z^{\bullet}$ by Fe(II) and Mn(II) are one and the same. The high specificity of the donation site for Mn(II) (*21*) indicates that it is associated with the native Mn binding site and also suggests that iron cations bind to the same native Mn binding site.

It is known that Mn-depleted PSII membranes are sensitive to donor side photoinhibition due to the action of strong oxidants such as $Y_Z^{\bullet}$ and $P680^+$ that are blocked from reduction by electrons from the tetrameric Mn cluster (*23*, *35*, *60*−*62*). The HA_Z Mn site is inhibited by weak illumination in parallel with both the decrease in $Y_Z \rightarrow P680^+$ electron transport and photodamage to Y_Z itself ($t_{1/2} \approx 2$ min) (*63*), as probed by the DPC-inhibition assay (*35*). These data alerted us to the possibility that the loss of electron donation capacity from Fe(II) to $Y_Z^{\bullet}$, following incubation of Mn-depleted PSII with Fe(II) under room light conditions (Figure 3), could be due to photodamage of Y_Z. However, in a control experiment, we found that Mn-depleted PSII membranes did not lose all of their donation sites during incubation for the same amount of time under room light in the absence of either Mn(II) or Fe(II) (compare Table 1, rows 1 and 2). These results support the notion that the observed loss of electron donation to $Y_Z^{\bullet}$ in Fe(II)-treated membranes is indeed due to irreversible binding of iron cations to the membranes and not to photodamage of Y_Z. Exogenous PSII electron donors are known to protect PSII from donor side photoinhibation. Thus, the absence of photodamage when Mn-depleted PSII membranes are incubated with Mn(II) in the light (Figure 3), as well as limited damage in the presence of Fe(III) and light (Table 1, rows 6 and 7), also demonstrates that the Fe-blocking effect is not the result of photodamage. Finally, the blocking effect was further confirmed by steady-state electron transport experiments in the absence of DCMU (Table 2). Photoreduction of DCIP supported by Mn(II) in Mn-depleted PSII membranes decreased to 25% (from 16 to 4) in membranes preincubated with Fe(II). Considering that bound iron totally inhibits donation through $Y_Z^{\bullet}$, we can assume that the remaining Mn(II) ⇒ DCIP activity is the donation of electrons by Mn(II) through the LA site. This assumption is supported by the kinetic data of Ghirardi et al. (*39*).

Since the Fe-blocking effect requires preillumination of the membranes (compare Table 1, rows 3 and 4), we suspected that it was dependent on the occupancy of the HA_Z site by a higher oxidation state of Fe [perhaps Fe(III)] but not by Fe(II). Previous work on the inhibition of DPC-supported DCIP photoreduction by added Fe(II) (*45*) supports this hypothesis. In that work, reactivation of DPC oxidation, following incubation with Fe(II) in the light, required treatment of the membranes with sodium dithionite, a strong reductant. This indicated that blocking of DPC photooxidation was affected by bound Fe. We tested this hypothesis by pretreating Mn-depleted PSII membranes for 5 min in the light with either Fe(II) or Fe(III) and then measuring the decay in the flash-induced fluorescence yield in the presence of added Mn(II) or Fe(II). In the experiments using exogenous Fe(III), we used a solution of $FeCl_3$ at pH 6.5. The iron was stabilized by addition of sucrose, which does not allow hydroxide [$Fe(OH)_3$] to form (*54*) but also does not prevent the interaction of exogenous Fe(III) with nonspecific binding sites on PSII (*64*). As seen in Table 1 (compare row

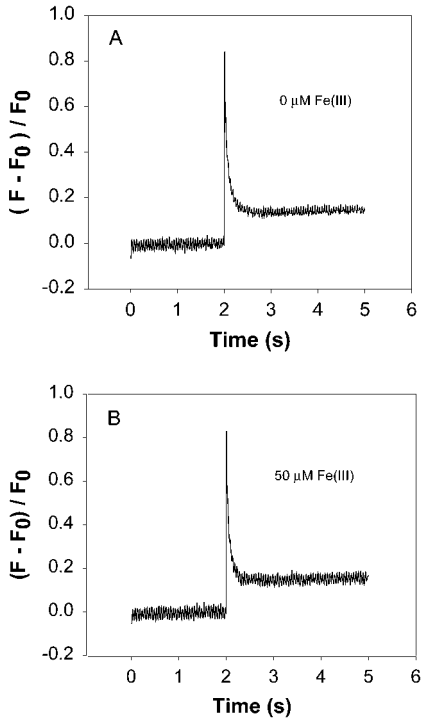

FIGURE 4: Effect of Fe(III) cations on the decay of the flash-probe fluorescence yield in Mn-depleted PSII membranes. $FeCl_3$ (50 μM) was added to the samples from a 1 mM stock solution of $FeCl_3$ stabilized with 0.6 M sucrose. See legend to Figure 1 and the text for other details.

7 with rows 2 and 3), exogenous Fe(III) was unable to block electron donation to $Y_Z^\bullet$, even though PSII membranes do bind the exogenous Fe(III) (*64*−*66*). Nonspecific binding of Fe(III) to Mn-depleted PSII membrane has been demonstrated by EPR (see ref *65* for a review) and Mössbauer spectroscopy (*64*, *66*). Moreover, addition of exogenous Fe(III) to Mn-depleted PSII membranes did not delay the kinetics of the flash-induced fluorescence decay, as seen in Figure 4 (compare with Figure 3). These results show that (i) exogenous Fe(III) binds nonspecifically and does not donate electrons to the HA_Z donation site, (ii) a *specific binding site* for photooxidized Fe is generated upon illumination of Mn-depleted membranes treated with Fe(II), and (iii) binding sites for exogenous Fe(III) and photooxidized Fe generated during oxidation of Fe(II) are different.

At this point we examined both the concentration and time dependencies of the Fe-blocking effect. Figure 5 shows a titration of the amount of the remaining electron donation capacity (the F_{fin}/F_{max} ratio) in Mn-depleted PSII membranes as a function of the initial Fe(II) concentration in the buffer. After incubation under room light, the membranes were centrifuged to remove unreacted iron, and electron donation to $Y_Z^\bullet$ by added 10 μM Fe(II) was measured using flash probe fluorescence. The blocking of the HA_Z Mn site follows a sigmoidal-type curve and reaches 100% at $\geq$2 μM Fe(II). This value corresponds to about 18 Fe(II) cations per reaction center of PSII (based on 220 mol of Chl per RC). In reality, blocking of the HA_Z site by Fe(II) occurs over a narrow range of Fe(II) concentrations (0.5−2 μM). Furthermore, the concentration of iron producing 100% blocking decreases with increasing light intensity during blocking (data not shown). Figure 6 shows the time dependence of the blocking effect. Blocking was measured as in the previous experiment,

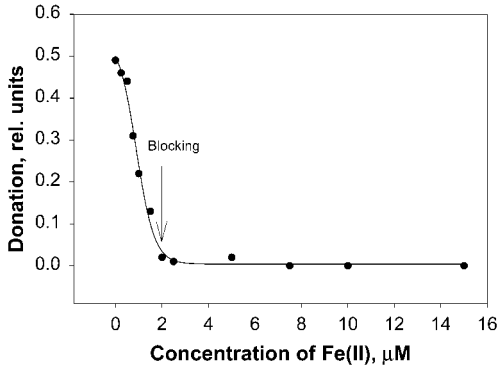

FIGURE 5: Titration of the blocking effect as a function of Fe(II) concentration. Mn-depleted PSII membranes (25 μg of Chl/mL) were incubated with different amounts of Fe(II) in buffer A (pH 6.5) under cool white fluorescent light (4 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) for 2 min. The samples were then centrifuged to remove any unreacted iron, and the reduction of $Y_Z^\bullet$ by added 10 μM Fe(II) was measured by flash-probe fluorescence. The amount of blocking was estimated as the amount of electron donation by re-added iron cations ($F_{fin}^{+10\,\mu M\ Fe(II)} - F_{fin}^{without\ Fe(II)}/F_{max}$). Fe(II) cannot donate electrons to $Y_Z^\bullet$ if the HA_Z site is totally blocked.

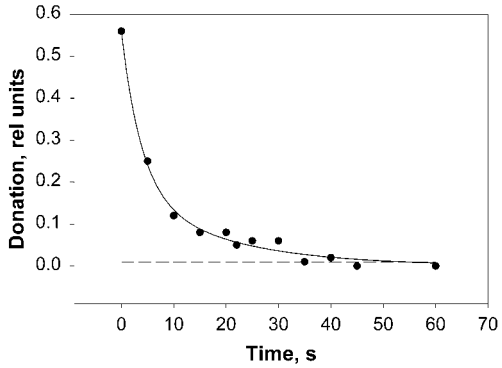

FIGURE 6: Time dependence of the Fe blocking effect measured with 10 μM Fe(II) as the electron donor. See Figure 5 legend and the text for additional details.

except that the incubation time was varied at constant initial Fe(II) concentration (10 μM). The results show that total blocking takes place in 40 s, a rather short period of time.

The results in Figure 2 and experiments using the DPC-inhibition assay (*45*) indicate that the Fe-blocking effect is characterized by the specific binding of more than one iron. To further examine this assertion, we measured both the pH dependence of electron donation by added Mn(II) to $Y_Z^\bullet$ via the HA_Z site and the pH dependence of the Fe-blocking effect. Since the stability of Fe(II) in solution depends strongly on pH, we did control experiments to make sure that saturating levels of Fe(II) were available at every pH. This entailed treating Mn(II)-depleted PSII membranes with 20 μM Fe(II) in MES buffer at pHs >6.5 and determining the remaining amount of Fe(II) in the solution using the *o*-phenanthroline assay after a 3 min incubation. Three minutes is enough time to do the Fe-blocking experiment at a saturating Fe(II) level. The results showed that at pH 8.0 about 40% of the Fe(II) still remained in solution. The pH dependencies are presented in Figure 7. The efficiency of Mn(II) donation through the HA_Z site increases with increasing pH, and the p*K* of this process is around 5.2 and saturates at pH = 6.0. On the other hand, the pH dependence of the Fe-blocking effect exhibits a very different pH profile. Formation of the Fe-blocking site also increases with

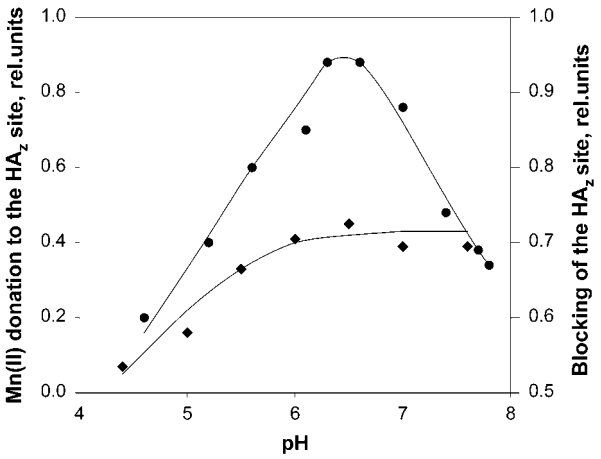

FIGURE 7: pH dependence of Mn(II) donation to the HA_Z site (◆) and blocking of the HA_Z site by iron cations (●). Electron donation by Mn(II) was determined using the flash-probe fluorescence decay method. Donation was estimated as $(F_{fin}^{+2.5\ \mu M\ Mn(II)} - F_{fin}^{without\ M_n(II)})/F_{max}$ (relative units). The efficiency of HA_Z site blocking by iron cations was estimated as electron donation to $Y_Z^•$ by 10 μM Mn(II) after the HA_Z site was blocked. This was calculated as follows: $[F_{max} - (F_{fin}^{+10\ \mu M\ Mn(II)} - F_{fin}^{without\ Mn(II)})]/F_{max}$ (relative units). See Materials and Methods for the Fe-blocking conditions.

increasing pH but reaches a maximum at about pH 6.4. At higher pHs the efficiency of HA_Z site blocking by specifically bound Fe decreases again. Comparison of these two curves shows that very different phenomena occur at higher pH.

As we have shown above, specifically bound Fe blocks further electron transfer from either Fe(II) or Mn(II) through the HA_Z Mn site. Figure 8A shows that the addition of 100 μM DPC to control Mn-depleted membranes reduces the extent of decay of the flash-induced fluorescence yield due to efficient electron donation from DPC to $Y_Z^•$ as reported earlier (*33*). In contrast, addition of 400 μM DPC to the same membranes with an Fe-blocked HA_Z site (Figure 8B) results in full decay of the fluorescence yield to the control level. This means that DPC cannot reduce $Y_Z^•$ in these samples and demonstrates that blocking the HA_Z site with iron also blocks the donation of electrons by DPC to PSII (see Discussion for more details). However, blocking the HA_Z site does not prevent the donation of electrons by added 1 mM hydroxylamine to $Y_Z^•$ (compare panels A and B of Figure 8). The lack of an Fe-blocking effect on the reduction of $Y_Z^•$ by this donor may be due to either (a) the reduction of Fe [≥Fe(III)] bound to the HA_Z site by hydroxylamine, followed by its release and resultant unblocking of the HA_Z site for electron donation to $Y_Z^•$ by another molecule of hydroxylamine, or (b) the existence of another donation site for hydroxylamine on PSII that does not overlay with either the HA_Z Mn or the DPC sites.

Hydroxylamine is often used to extract Mn from the OEC (*15*, *16*, *21*, *35*), which occurs as the result of the reduction of manganese ions in the OEC to Mn(II) (*67*). However, the effect of hydroxylamine on Fe ions bound to PSII is not known. Although the midpoint redox potential of the Fe(II)/Fe(III) couple [assuming that Fe(III) is the bound oxidation state] can be significantly lower than that of the Mn(II)/Mn(III) couple due to stabilization of Fe(III) by coordination with protein side groups, it is still possible that hydroxylamine can reduce and extract it from PSII as well

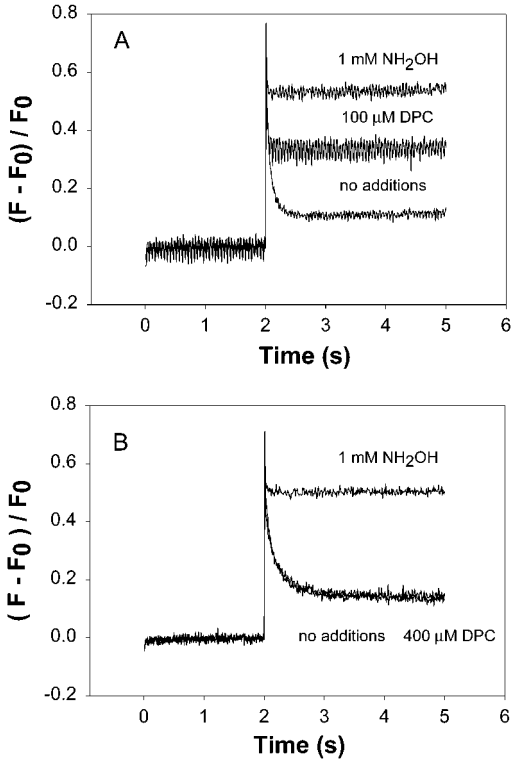

FIGURE 8: (A) Donation of electrons by 1 mM NH_2OH or 100 μM DPC to $Y_Z^•$ in Mn-depleted PSII membranes, as detected by the flash-probe fluorescence technique. Both donors were added just prior to flash excitation. Tris-washed (Mn-depleted) PSII membrane fragments at 25 μg of Chl/mL in buffer A (pH 6.5) were exposed to a saturating single-turnover flash in the presence of 37.5 μM DCMU. The samples were dark adapted before each measurement. (B) Effects of the addition of 400 μM DPC or 1 mM NH_2OH on the fluorescence decay in PSII membranes with an Fe-blocked HA_Z site. Mn-depleted PSII membranes (also 25 μg of Chl/mL) were blocked by preincubating them in buffer A with 5 μM $FeSO_4$ for 5 min under room light. Three measurements were averaged. See the text for other experimental conditions.

(see option a in the paragraph above). To investigate this possibility, Mn-depleted PSII membranes with an Fe-blocked HA_Z site were incubated with 1 mM hydroxylamine for 5 min in the dark. Such treatment is enough to extract manganese from the OEC of PSII depleted of the 16 and 23 kDa proteins (*17*). After incubation, the membranes were pelleted and washed to remove any residual hydroxylamine. Figure 9 shows that exogenous Mn(II) was unable to donate electrons to $Y_Z^•$ in these samples, indicating that the HA_Z site was still blocked by photooxidized Fe. Added NH_2OH, on the other hand, still donated to $Y_Z^•$. Thus, we conclude that NH_2OH does not release the bound Fe by reducing it; rather, it donates electrons to $Y_Z^•$ in Fe-blocked PSII via its own donation site (option b above) or, alternatively, it may donate electrons directly to $P680^+$.

DISCUSSION

Our results demonstrate that Fe(II) can donate electrons to $Y_Z^•$ in Mn-depleted PSII membrane fragments with an efficiency comparable to that of Mn(II) (see Figures 1 and 2). The fact that both Mn(II) and Fe(II) electron donations to $Y_Z^•$ saturate at about the same concentration is indirect evidence that they coordinate to the same site. Stronger evidence for this conclusion is the electron transfer blocking

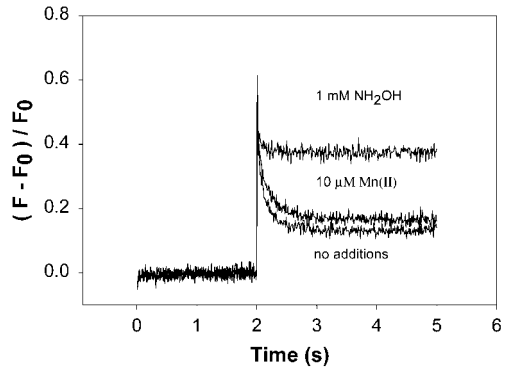

FIGURE 9: Influence of hydroxylamine on the Fe-blocked HA_Z Mn donation site. Mn-depleted PSII membranes (25 μg of Chl/mL) were incubated in buffer A with 5 μM $FeSO_4$ for 5 min under room light, after which the membranes were treated with 1 mM NH_2OH for 5 min in the dark. Hydroxylamine was then removed by centrifugating and washing the membranes, and the effects of added 10 μM Mn(II) and 1 mM NH_2OH on the flash-probe fluorescence yield decays were measured in the presence of 37.5 μM DCMU.

effect on Mn(II) oxidation by $Y_Z^{\bullet}$ when a photooxidized Fe cation(s) is (are) irreversibly bound specifically to the PSII membrane (Figure 3 and Table 2). Experiments in which a chemical modifier of carboxylic amino acid groups (EDC) was used to study effects of Mn on the yields of flash-induced fluorescence have demonstrated inhibition at the HA_Z donation site, but donation with lower efficiency was still present (*30*, *33*). This result was interpreted as evidence for the existence of high-affinity Mn ligands on PSII other than carboxyl residues that were "uncovered" following EDC treatment. In contrast, specifically bound Fe totally inhibits (Figure 3) the donation of electrons by Mn(II), suggesting the blocking of all HA_Z ligands to Mn(II) and further indicating that Fe cations ligate at the HA_Z Mn-donation site. Finally, the mutually exclusive interaction of both Fe(II) and Mn(II) with the site of inhibition of DPC donation (*45*) is also strong evidence that Fe(II) donates electrons through the HA_Z Mn-donation site.

Plots of F_{fin}/F_{max} as a function of Mn(II) or Fe(II) substrate concentration show a sigmoidal relationship for iron donation (Figure 2), which is characteristic of an allosteric enzyme effect. This means that the coordination of a first iron cation is necessary for coordination of a second. The same effect has been found using DPC-inhibition assay kinetic analyses, which shows that the inhibition of DPC photooxidation is determined by the cooperative binding of more than one iron cation (*45*).

The pH measurements (Figure 7) also suggest that more than one iron cation is involved in blocking of the HA_Z Mn site. Clearly, above pH 6.0, the pH dependence of the Fe-blocking effect is different than the pH dependence for Mn(II) electron donation via the HA_Z site. This can be interpreted as evidence for the involvement of an additional site for binding iron during the formation of the Fe block. We are unable to explain our data as an effect of high pH on other components of the electron transport chain since the membranes used for the two measurements shown in Figure 7 were the same. The declining pH dependence of the blocking effect at high pH might indicate that, in addition to a carboxylic residue with a p*K* of about 5.4, the protonated form of a basic residue is also essential for the irreversible binding of iron in PSII. Deprotonation of this group prevents the binding of an iron cation to this site. This second residue can represent a newly created site located close to the HA_Z site or part of a modified HA_Z site. In another words, these results again suggest that the Fe-blocking effect involves more than one specifically bound Fe. Furthermore, it is highly significant that the pH dependence of the Fe-blocking effect closely resembles the shape of the pH dependence of Mn -cluster reassembly during photoactivation (*14*, *15*) and O_2 evolution by PSII membranes (*68*), both of which require multiple metal ions.

An alternative interpretation of the pH data in Figure 7 (not requiring a second site at or near the HA_Z site) might involve differences in the pK_a's of the hydrated Mn and Fe species. Whether or not this is true for donation cannot be easily examined experimentally since the pH profile for Fe(II) donation to Y_Z at the HA_Z site requires one to work with exactly the same concentration of Fe(II) at each pH. This is particularly difficult at pHs above 6.5, because the cation is not stable above this pH. However, possible differences in p*K*'s do not seem to be relevant for Mn and Fe cation blocking at the PSII HA_Z site since again the pH profiles for the photoactivation of O_2 evolution at pHs below and above 6.4 are very similar to the pH profile for Fe blocking of Mn(II) donation. All the above observations imply that the light-dependent interaction of Fe(II) with the HA_Z Mn site is accompanied by the cooperative binding and oxidation of at least two iron cations. Finally, using the data shown in Figures 5 and 6 and other preliminary results obtained by measuring the rates of Fe(II) oxidation at the HA_Z site where the amounts of remaining unoxidized Fe(II) were measured (data not shown), we can place an upper limit on the number of oxidized iron cations at four to five cations per reaction center. Thus, at this point we can say that the number of iron forming the Fe block is between two and four to five.

The formation of a dinuclear Mn(III,III) center occurs under photoactivating conditions, which require the presence of Ca^{2+} and an acceptor. The initial steps of the photoactivation process, ultimately leading to the O_2 evolution capacity of PSII, involve the sequential binding and photooxidation of two Mn(II) cations. These two cations form a stable dinuclear Mn(III)−Mn(III) cluster in a process called photoligation. It is important to note that the transition from a mononuclear to a dinuclear center requires a conformational change that occurs in the dark. While photoactivation of O_2 evolution does not occur when Fe(II) is substituted for Mn-(II) (*69*), it is possible that the ligation of more than one iron during photooxidation of Fe(II) at the HA_Z site may be similar to the photoligation process for Mn. However, the quantum efficiency of a purported dinuclear iron center, if this is the blocking species formed, must be higher, considering that the rate-limiting step of Mn dinuclear center formation is dark conformational rearrangement (*15*) and that Fe can block Mn(II) donation. Manganese cannot block donation under the conditions applied in Table 2. This accounts for the low quantum efficiency of photoactivation (*15*) and suggests that a photoligation step could be more efficient in the case of iron (Figure 3).

If the photooxidation of Fe(II) leads to formation of a stable diiron cluster in a process analogous to the first two steps of the Mn photoactivation process, questions immediately arise about the oxidation state of the irons that

block the HA_Z site. Again, by analogy to the Mn case in PSII, one would suggest that an Fe(III)−Fe(III) cluster might form. While it is possible that higher iron oxidation states are involved in the blocking effect, Fe(IV−VI) states are rare in chemistry (*70*) and with the possible exception of proposed transient states in cytochrome P-450 are not known in biological systems. If such highly oxidized transient states exist in PSII, their formation is limited by the ca. +1.1 V midpoint potential of the $P680^+$/P680 couple in PSII. Finally, other diiron−oxo enzymes mentioned in the introduction are charcterized by Fe(III)−Fe(III) clusters (*48*, *71*) associated with the same residues and motifs observed on the D1 and D2 proteins (see also the discussion below). Therefore, we suggest that in the absence of any other information at this point an Fe(III)−Fe(III) diiron cluster is the most likely species that blocks the HA_Z Mn site.

At least one specifically bound Fe(III) that blocks the HA_Z site must be located close to Y_Z, since its formation causes the recombination rate of $Y_Z^\bullet$ with Q_a^- to decrease (Figure 3). However, the purported Fe(III) cations that appear as the result of Fe(II) oxidation at the HA_Z site bind to a site that is different from those binding exogenous Fe(III). This is clear because Mn(II) donates in the presence of exogenous Fe(III) (Table 1, row 6) and because exogenously added Fe(III) has no influence on the kinetics of fluorescence decay (Figure 4). This means that a stable Fe [≥Fe(III)] binding site is created during the oxidation of Fe(II) at the HA_Z site. As mentioned above, the same mechanism for the creation of Mn(III) binding sites takes place during the photoassembly of the Mn cluster (*21*), and this fact reemphasizes the similarity between the effects of Mn(II) and Fe(II).

Previous experiments have suggested that DPC donates an electron to $Y_Z^\bullet$ through a site other than the HA_Z Mn donation site: (a) Lineweaver−Burke studies show non-competitive interaction between Mn(II) and DPC with the donor side of Mn-depleted PSII membranes using the DPC-inhibition assay (*38*); (b) Co^{2+} and Zn^{2+} inhibit flash-induced photooxidation of DPC but not of Mn(II) (*39*); (c) EDC treatment of Mn-depleted PSII membranes inhibits the binding of Mn(II) to the HA_Z Mn site as detected by the DPC-inhibition assay but has little effect on DPC oxidation (*31*); and (d) EDC treatment inhibits electron donation to $Y_Z^\bullet$ by Mn(II) to a much greater extent than by DPC (*33*). In the current study, we have taken advantage of the irreversible blocking of the HA_Z Mn donation site by specifically bound Fe to further study high-affinity DPC donation to Mn-depleted PSII membranes. Previous studies outlined above show that Mn(II) [or Fe(II)] and DPC donate at different high-affinity sites. However, specifically bound Fe at the HA_Z Mn site also blocks DPC donation (Figure 8). Therefore, we conclude that the inhibition of DPC donation upon ligation of specifically bound Fe to the HA_Z Mn site must be caused by conformational changes in the protein upon Fe blocking that also prevent the donation of DPC to its own donation site.

The results reported in this paper also confirm that the LA donation site for DPC does not involve $Y_Z^\bullet$, since membranes in which electron donation to $Y_Z^\bullet$ by 400 μM DPC is blocked by specifically bound Fe (Figure 8A) still exhibit 50% of the electron transfer rate to the acceptor side of PSII as measured by DCIP photoreduction (*45*). This means that there is a second electron acceptor for DPC oxidation at the LA site and that the HA_Z blocking effect can be used to facilitate more detailed investigations of the LA site.

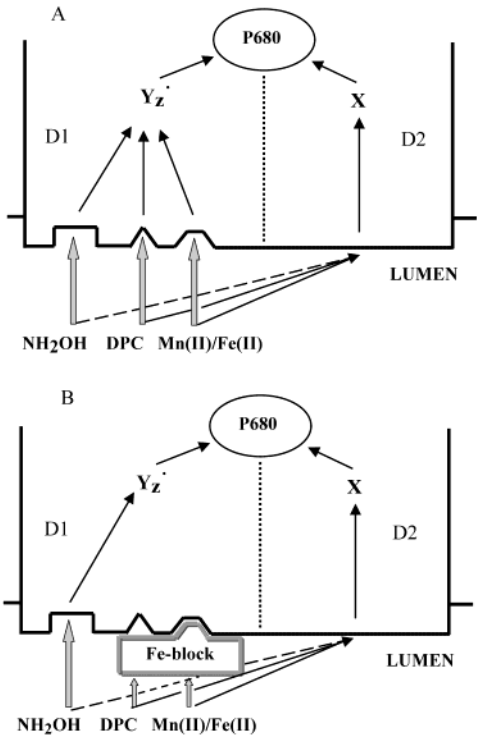

FIGURE 10: Schematic diagram summarizing the donation sites of several exogenous donors to the oxidizing side of Mn-depleted PSII membranes: (A) control conditions; (B) in the presence of the Fe(III) block. If X is $Y_D^\bullet$, then D2 is the location.

In contrast to DPC donation, NH_2OH donation is not affected by the presence of an Fe-blocked HA_Z site. We show that this is not due to the reduction and release of the bound Fe by NH_2OH (see Figure 9) but rather to the existence of a separate high-affinity site for hydroxylamine donation to $Y_Z^\bullet$ on PSII. Alternatively, NH_2OH might donate electrons directly to $P680^+$. In any case, our current results demonstrate that the hydroxylamine donation site does not coincide with either the DPC or the HA_Z Mn donation sites. Figure 10 is a scheme summarizing the above information about donor sites and the effect of Fe blocking of the HA_Z Mn site.

Of particular significance in support of the idea that a diiron cluster can form in our system is the fact that the C-termini of the D1 and D2 proteins found in the PSII RC both contain conserved motifs and residues that should be able to bind and stabilize two irons (*50*). These motifs were identified by comparing the primary amino acid sequences of D1 and D2 with those of known diiron−oxo enzymes, including ribonucleotide reductase (the R2 component), methane monooxygenase and desaturase, which exhibit oxygen-activation function. X-ray crystal structures of these proteins confirm that the binding of two irons is associated with these residues and motifs (*72*, *73*) and that Mn can substitute for the irons (*48*, *49*). On the basis of the similarity between the interaction of Mn(II) and Fe(II) with the HA_Z site detected by the DPC-inhibition assay (*44*, *45*), it was proposed that these motifs and residues (D319, E329, H332, and E333 on the D1 protein and E324, D334, H337, and E338 on the D2 protein) participate in the coordination (D319, E329, and H332 on D1 and E324, D334, and H337

on D2) and stabilization (E333 on D1 and E338 on D2) of the dimanganese cluster that forms during the assembly of the tetranuclear Mn cluster. The fact that both Mn(II) and Fe(II) interact directly with the HA_Z Mn site (the current study) not only further strengthens the above hypothesis but also supports the possible formation of a diiron cluster in PSII.

Indeed, some of the above residues have been implicated in Mn-catalyzed water oxidation activity. Site-directed mutagenesis of conservative carboxylate residues and histidines on the lumenal sides of the D1 and D2 subunits has shown that H332, E333, and H337 on D1 influence the assembly and/or stability of the tetrameric Mn cluster (*74*). Participation of H337 in the binding of Mn(II) at the HA_Z Mn site was also proposed earlier on the basis of experiments with amino acid modifiers and carboxypeptidase A (*20*). Mutation of the two other residues on D1 (D319E and E329D) that have been proposed to coordinate Mn (*50*) have not influenced O_2 evolution (*74*). However, substitution of one carboxylic residue by another may not affect coordination. For example, substitution of Asp-170, which probably is a ligand to the photooxidizable Mn (*22*, *33*, *40*, *57*), by glutamate did not affect the rate of O_2 evolution in the resulting mutants (*40*). On the other hand, the D1-H332E mutant does not evolve O_2 but does assemble Mn clusters in nearly all PSII reaction centers (*74*, *75*). Furthermore, O_2 evolution was not influenced in D1-D319N and D1-E329Q mutants (*74*); however, the participation of the carbonyl groups of N and Q in the coordination of Mn cannot be excluded.

The participation of the C-terminus of D2 in Mn coordination has been controversial. Early site-directed mutagenesis studies of conservative carboxylate residues and histidines on the lumen side of D2 subunit led to the conclusion that these residues do not provide ligands to Mn (*76*). However, experiments with truncated D2 protein indicate that the C-terminal domain may have a role in O_2 evolution (*77*). A role for C-terminal residues of D2 in Mn cluster coordination was also suggested on the basis of symmetry arguments (*34*).

Finally, participation of the above-indicated motifs and residues in the ligation of the Mn cluster should be examined more carefully, since they are similar to those found in diiron−oxo enzymes and since the present study shows distinct similarity in the primary ligation steps of Fe and Mn in PSII. Regardless of their participation in the formation of the OEC, these motifs are interesting from an evolutionary perspective. They are located on the carboxyl ends of D1 and D2 in the PSII reaction center, which are significantly larger than the carboxyl ends of the analogous L and M subunits in the bacterial reaction center (which does not evolve O_2). Perhaps the motifs that formed diiron catalytic centers in primitive organisms participated in the oxidation of substrates other than water, or bound Fe(II) served as a substrate for oxidation in the distant past. Iron(II) has been proposed recently as a likely candidate as an early electron donor in Archean oceans (*78*).

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