A carboxylic residue at the high-affinity, Mn-binding site participates in the binding of iron cations that block the site

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Abstract

The role of carboxylic residues at the high-affinity, Mn-binding site in the ligation of iron cations blocking the site [Biochemistry 41 (2000) 5854] was studied, using a method developed to extract the iron cations blocking the site. We found that specifically bound Fe(III) cations can be extracted with citrate buffer at pH 3.0. Furthermore, citrate can also prevent the photooxidation of Fe(II) cations by $Y_Z^{\bullet}$. Participation of a COOH group(s) in the ligation of Fe(III) at the high-affinity site was investigated using 1-ethyl-3-[(3-dimethylamino)propyl] carbodiimide (EDC), a chemical modifier of carboxylic amino acid residues. Modification of the COOH groups inhibits the light-induced oxidation of exogenous Mn(II) cations by Mn-depleted photosystem II (PSII[−Mn]) membranes. The rate of Mn(II) oxidation saturates at $\geq 10\ \mu M$ in PSII(−Mn) membranes and $\geq 500\ \mu M$ in EDC-treated PSII (−Mn) samples. Intact PSII(−Mn) membranes have only one site for Mn(II) oxidation via Y_Z (dissociation constant, $K_d = 0.64\ \mu M$), while EDC-treated PSII(−Mn) samples have two sites ($K_d = 1.52$ and $22\ \mu M$; the latter is the low-affinity site). When PSII(−Mn) membranes were incubated with Fe(II) before modifier treatment (to block the high-affinity site) and the blocking iron cations were extracted with citrate (pH 3.0) after modification, the membranes contained only one site ($K_d = 2.3\ \mu M$) for exogenous Mn(II) oxidation by $Y_Z^{\bullet}$ radical. In this case, the rate of electron donation via Y_Z saturated at a Mn(II) concentration $\geq 15\ \mu M$. These results indicate that the carboxylic residue participating in Mn(II) coordination and the binding of oxidized manganese cations at the HA_Z site is protected from the action of the modifier by the iron cations blocking the HA_Z site. We concluded that the carboxylic residue (D1 Asp-170) participating in the coordination of the manganese cation at the HA_Z site (Mn4 in the tetranuclear manganese cluster [Science 303 (2004) 1831]) is also involved in the ligation of the Fe cation(s) blocking the high-affinity Mn-binding site.
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1. Introduction

Photosynthetic water oxidation in plants, green algae and cyanobacteria is accompanied by the synthesis of an intermolecular bond between the oxygen atoms of two water molecules and the subsequent evolution of molecular O_2 into the atmosphere. This reaction is catalyzed by the photosynthetic O_2-evolving complex (OEC), located within the photosystem II (PSII) membrane. The OEC is composed of a tetranuclear manganese/mononuclear calcium/oxo cluster that also needs at least one Cl ion for catalytic activity [1–7].

The structure of the $(Mn)_4/Ca$ cluster and the composition of the ligands responsible for coordinating the cluster have been the subject of many investigations. Despite remarkable progress in the field of PSII structure and function, several issues are still

not fully resolved. The first X-ray structures of PSII at 3.8 Å and 3.7 Å resolution, published by Zouni et al. [8] and Kamiya and Shen [9], respectively, outlined a structural framework for PSII, but the exact arrangement of Mn and calcium ions was not resolved. Ferreira et al. [1] and Biesiadka et al. [2] solved the structure of PSII at 3.5 Å and 3.2 Å resolution, respectively, and the amino acid ligands to the Mn cluster were assigned. According to Ferreira et al. [1], the OEC consists of a cubane-like Mn_3CaO_4 cluster where each metal ion is associated with three μ-oxo bridges. A forth Mn ion is linked to the cubic structure by one of the oxo bridges. Most of Mn cluster ligands arise from the D1 reaction center (RC) polypeptide (including Asp170, Glu189, Glu333, His332, and Asp342), and one (Glu354) is associated with CP43.

Although all of the Mn ions in the cluster have amino acid ligands, only one Mn-binding site, the (HA_Z) Mn(II) site [10–14], can be detected experimentally in Mn/Ca-depleted PSII membranes. This site can coordinate an exogenous Mn(II) cation before it is oxidized by $Y_Z^\bullet$ (formed from primary charge-separation in the PSII RC), and it can be detected by monitoring Mn(II) oxidation. This Mn(II) oxidation reaction is regarded as the first step in the photoligation/photoactivation of the Mn cluster. Different assays have been used to detect manganese oxidation at this site, including (a) dichlorophenolindophenol (DCIP) photoreduction with Mn(II) alone or in the combination with hydrogen peroxide [10,12,14–21], (b) Mn(II)-inhibition of electron donation by diphenylcarbazide (DPC) [22–29], (c) Mn(II) effects on the yield and decay kinetics of chlorophyll (Chl) a fluorescence [30–34], and (d) optical spectroscopy [13] or EPR measurements of the rate of $Y_Z^\bullet$ radical reduction [11,20,33]. The value of K_d for Mn(II) coordination at the HA_Z site determined in these studies varied from around one to several micromolar [12–14,20,22,25, 28,31,33]. Ono and Mino [33], monitoring the $Y_Z^\bullet$ radical EPR signal, also measured the amounts of Mn(II) bound to PSII(−Mn) membranes, thereby proving directly that PSII membranes have only one unique high affinity/high specificity site for Mn(II) responsible for reducing oxidized $Y_Z^\bullet$ radical.

Chemical modification and site-directed mutagenesis were originally used in attempts to identify Mn(II) ligands at the HA_Z site. Experiments with the chemical modifier, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC), showed that a carboxylic residue participates in the coordination of Mn(II) cations [19,20,21,25,31]. Several mutagenesis and spectroscopic studies revealed that D1-Asp170 might be the carboxylic residue [13,30,31,35–38]. A dual-mode EPR study suggested that D1-Asp170 directly ligates the Mn(II) cation at the HA_Z site [39]. However, these investigations did not determine whether D1-Asp170 participates only in the coordination of Mn(II) that is photooxidized during the initial photoactivation step or if it also ligates the assembled $(Mn)_4$ cluster [40,41]. The recent PSII structure [1,2] resolved this issue and showed that D1-Asp170 does coordinate the assembled $(Mn)_4$ cluster; however, the Mn cation ligated by this amino acid residue does not change valency during S-state transitions [42].

Besides Mn(II) ions, the HA_Z, Mn-binding site can also ligate a number of another metal cations. Hsu et al. [22] and

Ghirardi et al. [28], using the DPC-inhibition assay, found that HA_Z site can coordinate Co(II) and Zn(II), but with much less efficiency (K_i=18 and 33 μM, respectively [28]) than Mn(II) (K_d=0.94 μM [31]), demonstrating that the interaction of these ions with the HA_Z site was non-specific. Mg(II) and Ca(II) are not coordinated in micromolar concentrations [22]. At millimolar concentrations, Ca, Ba, Zn, Cu, Mg and Co cations can protect the HA_Z site against the action of EDC, but only partially (50%) [25]. From the point of view of specificity, iron cations are quite interesting. The DPC-inhibition assay indicates that Fe(II) cations bind to the HA_Z site in the same concentration region as Mn (about 1 μM) [27], and interaction between Mn(II) and Fe(II) at this site is mutually exclusive [29]. Moreover, binding of iron cations is a light-dependent process [27]. Incubation of Mn-depleted PSII membranes with Fe(II) under photoactivating conditions (weak light for 30 s to 2 min, depending on the iron concentration and light) causes the irreversible binding of Fe cations to the HA_Z site and the "blocking" of this site (i.e., exogenous Mn(II) ions, Fe(II) cations, and DPC cannot donate electrons to $Y_Z^\bullet$ if the samples are blocked with iron) [34]. At least two bound iron cations are required to produce the blocking effect, which can be observed only after 4–5 Fe cations are oxidized by $Y_Z^\bullet$ radicals [43]. The irreversible blocking of the HA_Z site is accompanied by a significant modification of the electron-transfer characteristics on the donor side of the PSII(−Mn) membrane. For example, the pK_{app} for $Y_Z^\bullet$ reduction during $Y_Z^\bullet$ $P680^+$ recombination shifts from 7.1 to 6.1 [43]. This is closer to the pK_{app} for electron transfer between Y_Z and P680 in the intact compare to PSII(−Mn) membranes [44]. In iron-blocked PSII(−Mn) membranes [45] as well as in intact membranes [46], Y_Z can be oxidized at much lower temperatures (<77 K) than in Mn-depleted membranes (>180 K) [47]. These observations can be explained by the transformation of the weak hydrogen bond between Y_Z and base B (D1-His190), seen in PSII(−Mn) samples, into a strong hydrogen bond with a low activation barrier seen in PSII (−Mn,+Fe) membranes [45]. Zhang and Styring [46] have postulated a low barrier hydrogen bond in intact membranes. Such reconstitution of PSII donor side properties by bound iron cations suggests that the iron cation(s) blocking the HA_Z site occupy one or several Mn-binding sites. The specific objective of this paper is to determine if the carboxylic residue at the HA_Z site (D1-Asp170) coordinates the iron cations that block the site as is the case for manganese.

2. Materials and methods

2.1. Biological samples

BBY-type, PSII-enriched membrane fragments were prepared from market spinach [34] and resuspended in buffer A (50 mM MES/NaOH, 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. [48]. The rate of O_2 evolution by the PSII membranes was 400–500 μmol of O_2 mg Chl^{-1} h^{-1}, and the membranes were stored under liquid nitrogen until use. Manganese-depletion was accomplished by incubating thawed PSII membranes (0.5 mg 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 [34].

2.2. Blocking of the HA_Z, Mn-binding site

PSII(−Mn) membranes (25 µg Chl/mL) were incubated in buffer A (pH 6.5) with (a) 10 µM $FeSO_4$ in experiments where the effects of citric acid on the blocking irons cations was studied or (b) 15 µM Fe(II) in experiments with EDC-treated membranes. Larger than normal concentrations of Fe(II) were used for blocking [34,43,45] to prevent the extraction of bound iron (10–15%) during citrate, EDC, and centrifuge-washing treatments. Incubation was done under cool white fluorescent room light (4 µmol quanta × m^{-2} × s^{-1}, PAR) for 2 min (in experiments with citric acid) or 1.5 min (in experiments with EDC) at room temperature. The membranes were then pelleted by centrifugation (15000×g, 10 min), and re-suspended in either buffer C (0.4 M sucrose, 20 mM NaCl, 10 mM citric acid, pH 3.0) for iron-extraction experiments or buffer A (pH 6.5) before EDC treatment.

2.3. Chemical modification

Chemical modification of carboxylic groups was carried out as described before [20,25,31] in buffer A, using 22 mM EDC and 0.25 mg Chl/mL PSII (−Mn) membranes. The modification reactions were run for 60 min in the dark at room temperature.

2.4. Flash-probe fluorescence measurements

The decay of the flash-probe fluorescence yield was measured at room temperature using a home-built instrument capable of 100 µs time resolution [28]. Samples, containing 25 µg Chl/mL (ca. 0.12 µM PSII centers) and 40 µM DCMU in buffer A, were dark-adapted prior to measurement. Data analysis used Data Translation Global Lab software and a DT2839 A/D board mounted in an ALR 486 PC.

3. Results

3.1. Extraction of blocking iron cations

As has been noted above, EDC treatment inactivates the HA_Z, Mn(II)-binding site by modifying a carboxylic residue(s) involved in the coordination of the Mn(II) cation [19–21,25,31]. If this carboxylic residue(s) participates in the binding of the blocking iron cation(s) (i.e., Fe(III) appearing during oxidation of Fe(II) bound to the HA_Z, Mn-binding site), then the iron should protect the ligand(s) from EDC modification, and this protective function should preserve the potential for high rates of Mn(II) oxidation via this site. Since the blocking iron does not allow electron donation via the HA_Z site by Mn(II), Fe(II) or DPC [34], the ability of Mn(II) to donate an electron to $Y_Z^\bullet$ must be assayed in EDC-treated PSII (−Mn,+Fe) samples only after extracting the blocking iron cations. To do this, we had to develop a method to extract the iron bound to the PSII(−Mn,+Fe) membranes.

We found previously that blocking iron cations can be extracted from the HA_Z binding site at alkaline pH's and that Tricine accelerates this process [43]. However, the release of Fe (III) into the medium at a pH > 2 should be accompanied by iron hydroxide formation due to its very high stability constant [49]. Although the presence of iron hydroxide in principal should not affect the results of our experiments, its presence can complicate data interpretation in some other types of studies (for example in experiments where iron is detected by γ-resonance [Mössbauer] techniques). Therefore, we used citrate treatment of the samples at low pH (3.0) as an alternative extraction method. Ono and

Inoue first suggested this method as a way to extract Ca ions from the OEC [50]. Citrate is an efficient chelator of ferric cations (K_{st}=11.85 [51]), and the treatment occurs at pH 3.0, which is less than the pK of the carboxylic groups (about 4.0) representing most of the ligands in the coordination sphere around the Mn cluster [1,2]. Presumably, these same groups surround the blocking iron cations as well [52], and therefore this method might also be suitable for iron extraction.

The results of our experiments with citric acid buffer are presented in Fig. 1 and Table 1. We studied electron donation by 10 µM Mn(II) to $Y_Z^\bullet$ upon monitoring the Chl a fluorescence yield decay kinetics elicited by a saturating flash as before [28,31,32,34,43,45] after citrate treatment of PSII(−Mn) and PSII(−Mn,+Fe) membranes. Fig. 1Ia and IIa demonstrate that blocking the HA_Z site prevents the donation of electrons by Mn(II) to $Y_Z^\bullet$ as we found earlier [34]. After citrate treatment of the blocked samples at pH 3.0, the HA_Z site becomes accessible for Mn(II) cation donation (Fig. 1IIb), and the efficiency of donation increases to the level characteristic of unblocked samples (Fig. 1 and Table 1). These results show that citrate extracts all of the iron cations blocking the HA_Z site and that the extraction process is rather fast (≤1 min; Table 1). Only the combination of two factors, pH and chelator, provides for the complete extraction of the blocking irons. Citrate at higher pH (6.5) does not fully extract iron cations as indicated by the fluorescence decay kinetics (compare Fig. 1IIc with IIb). Increasing the citrate concentration to 30 mM (Table 1) or more (80 mM, results not shown) does not influence the tightly bound iron pool. Although citrate binds ferric cations effectively (K_{st}=11.85), it is a significantly weaker chelator of ferrous cations (K_{st}=3.2). Nevertheless, Fe (II) cations cannot donate electrons to $Y_Z^\bullet$ in the presence of citrate (Table 1). This result indicates that the K_{st} for the Fe (II)–HA_Z site complex is probably less then 3.2. In summary, these results show that the blocking iron cation(s) can be fully extracted from blocked membranes by treating with citrate buffer at low pH (3.0), and this method was used in the following experiments.

3.2. EDC-treatment

In this section, we investigated electron donation by Mn(II) cations to $Y_Z^\bullet$ before and after EDC treatment. The efficiency of electron donation by Mn(II) to $Y_Z^\bullet$ as function of Mn(II) concentration is presented in Fig. 2 and can be represented by the equation [1−relative $(F_{max}−F_{final})/F_{max}$] [31]. The value "relative $(F_{max}−F_{final})/F_{max}$" is equal to the ratio [$(F_{max}−F_{final})/F_{max}$]$^{+Mn(II)}$/[$(F_{max}−F_{final})/F_{max}$]$^{−Mn(II)}$ and is a measure of the number of PSII centers that can recombine while the expression [1−relative $(F_{max}−F_{final})/F_{max}$] reflects the number of PSII centers that do not recombine due to the reduction of $Y_Z^\bullet$ by exogenous Mn(II). Since, the same PSII(−Mn) preparations were used in all experiments, the value [1−relative $(F_{max}−F_{final})/F_{max}$] can be used as an estimate of the relative number of active HA_Z donation sites [31], and hence, the relative amount of electron donation before and after EDC-treatment. The data from Fig. 2 were used to calculate the Scatchard plot
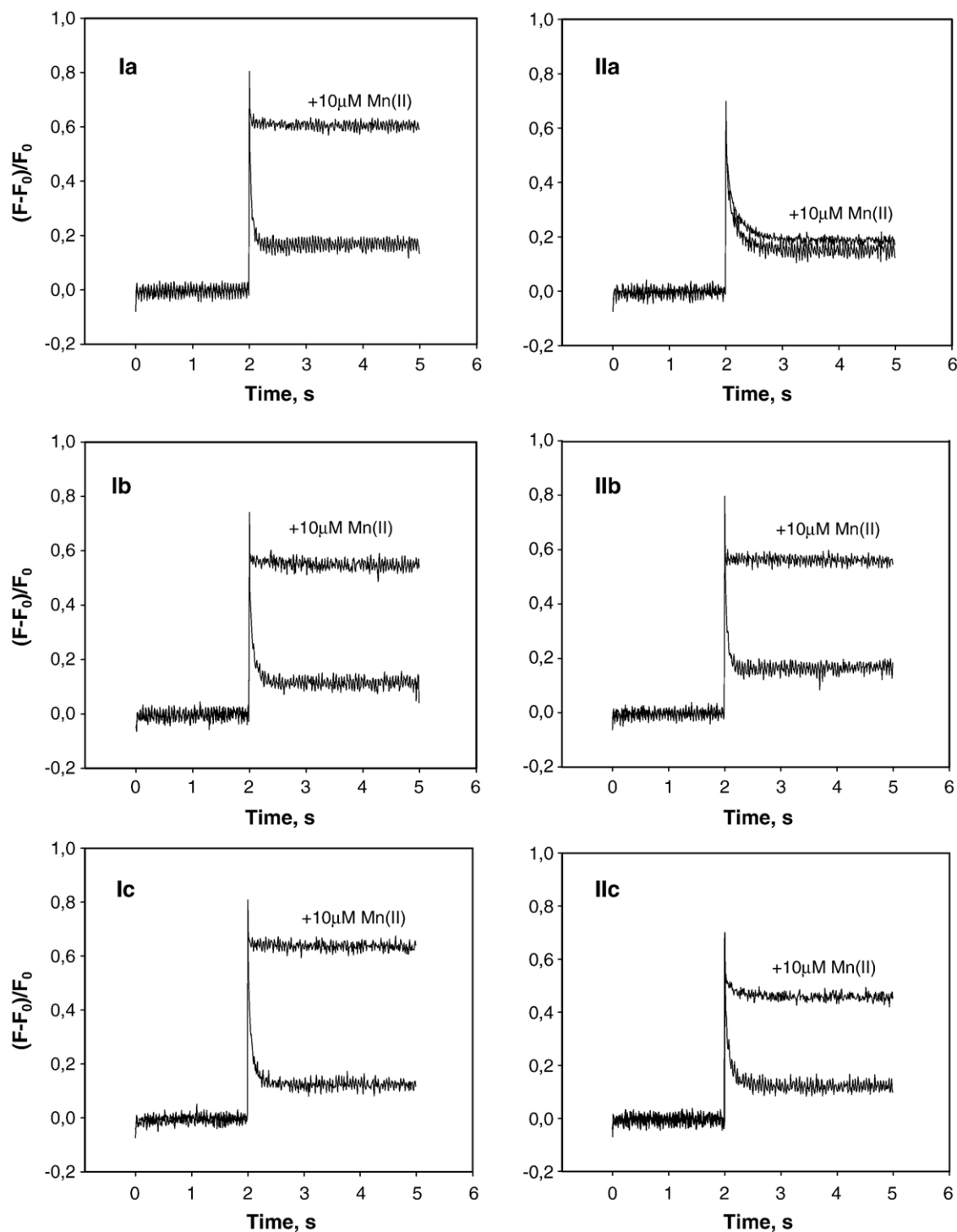
Fig. 1. Effect of citrate on the flash-probe fluorescence yield in Mn-depleted PSII membranes (I) and in Mn-depleted PSII membranes with its HA_Z, Mn-binding site blocked by iron cations (II). Samples: Ia and IIa, without citrate treatment; Ib and IIb, after incubation of the membranes (25 μg Chl/mL) for 5 min (dark, room temperature) in buffer C (400 mM sucrose, 20 mM NaCl, 10 mM citric acid, pH 3.0); Ic and IIc, after incubation of membranes (25 μg Chl/mL) for 5 min (dark, room temperature) in buffer A (400 mM sucrose, 20 mM NaCl, 50 mM MES, pH 6.5) with 10 mM citric acid. After citrate treatment, the samples were washed twice in buffer A and the flash-probe fluorescence yield was measured in buffer A (25 μg Chl/mL) in the presence of 40 μM DCMU with or without 10 μM $MnCl_2$.

shown in insert. The plot shows that Mn(II) has one binding site with a binding affinity K_d equal to 0.64 μM estimated from the slope of a straight line. This value is roughly consistent with the value 0.94 μM [31] and 1.3 μM [33] obtained by the same method.

The treatment of PSII(−Mn) membranes with EDC strongly inhibits electron donation by Mn(II) to $Y_Z^{\bullet}$ in the micromolar concentration range (Fig. 3), consistent with earlier reports [19–21,25,31], but $Y_Z^{\bullet}$ reduction can be observed at higher Mn(II) concentrations. The saturation of $Y_Z^{\bullet}$ reduction in EDC-treated

Table 1
Effect of citrate treatment on bound iron cations, blocking the HA_Z, Mn-binding site and on electron donation by Fe(II) to $Y_Z^{\bullet}$ in Mn-depleted PSII membranes

Sample	Citrate [a]			Donation [b] (rel. units)
	Incubation time (min)	pH	Concentration (mM)	
Effect on the iron cations blocking the HA site				
PSII(−Mn)	Not treated			0.52 ± 0.02
	5	3.0	10	0.57 ± 0.01
	5	6.5 [c]	10	0.63 ± 0.01
PSII (−Mn,+Fe)	Not treated			0.05 ± 0.01
	1	3.0	10	0.53 ± 0.01
	5	3.0	10	0.52 ± 0.01
	5	5.2	10	0.57 ± 0.01
	5	6.5 [c]	10	0.50 ± 0.07
	5	6.5 [c]	30	0.48 ± 0.03
Effect of citrate on the electron donation by 10 µM Fe(II)				
PSII(−Mn)	Not treated			0.58 ± 0.01
	0.5	6.5 [c]	10	0.04 ± 0.01

[a] Citrate buffer: 400 mM sucrose, 20 mM NaCl, citric acid as indicated (pH 3.0).
[b] Donation: $[(F_{max} - F_{final})/F_{max}]^{-Mn} - [(F_{max} - F_{final})/F_{max}]^{+Mn}$. Donation was determined in buffer A (25 µg Chl/mL of membranes) in the presence of 40 µM DCMU.
[c] Buffer A (400 mM sucrose, 20 mM NaCl, 50 MES, pH 6.5) + citric acid as indicated (pH 6.5).

membranes occurs at Mn(II) concentration > 500 µM, whereas in non-EDC-treated membranes, saturation occurs at about 10 µM. The Scatchard plot (see Fig. 3 insert) shows that chemical modification of the carboxylic residues leads to a decrease in the relative amount of HA_Z site (compare 0.84 in
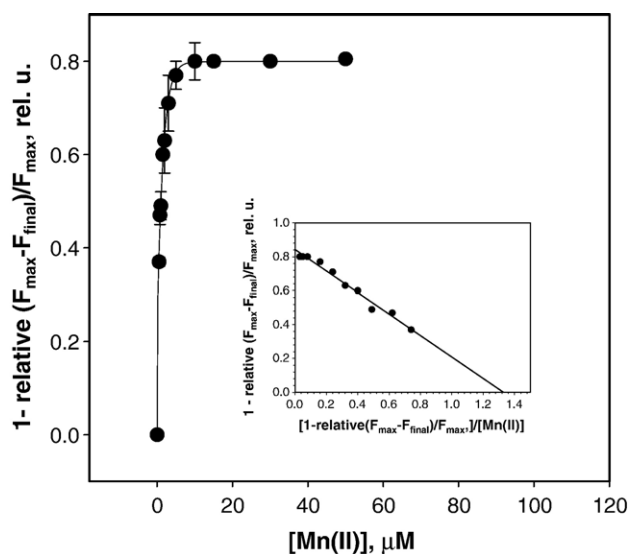

Fig. 2. Concentration dependence of electron donation by Mn(II) to $Y_Z^{\bullet}$ in PSII (−Mn) membranes. Electron donation was determined from flash-probe fluorescence decay kinetics and expressed as $[1 - \text{relative} \, (F_{max} - MnF_{final})/F_{max}]$ value (see Results for details). The fluorescence measurements were done in buffer A (pH 6.5) with 40 µM DCMU, and the concentration of membranes assayed was 25 µg Chl/mL. Each point on the figure is an average of three measurements on different samples. The experimental data (solid circles) were fitted with one exponential curve (solid line). Insert: Scatchard plot of the above data. The x- and y-intercepts were used to estimate the binding affinity, K_d, for Mn(II) at the HA_Z site and the relative amount of binding sites.
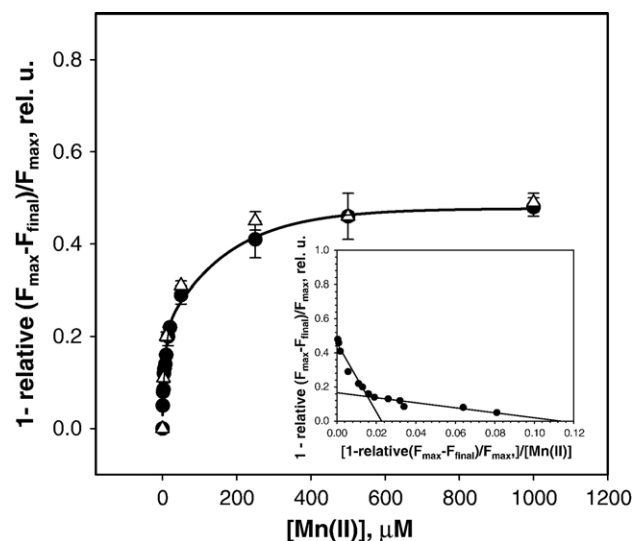
Fig. 3. Concentration dependence of electron donation by Mn(II) to $Y_Z^{\bullet}$ in EDC-treated PSII(−Mn) membranes (solid circles). The data were fitted with two exponentials (solid line). Note that x-axis scale differs from x-axis scales on Figs. 2 and 4. PSII(−Mn) membranes (0.25 mg Chl/mL) were dark incubated for 1h in buffer A at room temperature with 22 mM EDC. The membranes were then washed twice with buffer A, and the concentration dependence of electron donation by Mn(II) cations was measured. Some of the EDC-treated membranes were incubated for 1.5–2 min in citrate buffer C (pH 3.0), and after washing the samples, Mn(II) electron donation was measured in buffer A (open triangles). For other details see the Fig. 2 legend. Insert: Scatchard plot of the above data (Mn(II) donation after EDC-treatment). The x- and y-intercepts were used to estimate the binding affinity K_d for Mn(II) at the HA_Z site and the relative amount of binding sites.

PSII(−Mn) membranes (Fig. 2 insert) with 0.48 in EDC-treated membranes (Fig. 3 insert)), some increase in the K_d of the site (1.52 µM), and to the appearance of a low-affinity donation site (LA_Z) to $Y_Z^{\bullet}$ with $K_d = 22$ µM (relative amount 0.17, Fig. 3 insert). Also, the number of inactivated HA_Z sites ($Y_Z^{\bullet}$ cannot be reduced by Mn(II) via these sites) is increased after EDC treatment. In PSII(−Mn) membranes the number of such sites is equal 20% (Fig. 2) whereas EDC-treatment increases this value to 53% (Fig. 3). Thus, EDC-treated membranes have two donation sites to $Y_Z^{\bullet}$ instead of one in untreated membranes (Table 2). The appearance of the LA_Z site with $K_d = 22$ µM (but dependent on EDC concentration) in Mn-depleted PSII membranes after EDC modification was observed earlier [31].

Table 2
Effects of EDC-treatment and blocking of the HA_Z, Mn-binding site by iron on the Mn(II) donation sites to $Y_Z^{\bullet}$ and their binding affinities

Sample	Number of sites for Mn(II) donation to $Y_Z^{\bullet}$	K_d (µM)	Relative number of binding centers
PSII(−Mn)	1	0.64	0.84
PSII(−Mn) after EDC treatment	2	1.52	0.17
		22	0.48
Blocked PSII (−Mn) membranes were treated by EDC, after then blocking iron cations were extracted by citrate (pH 3.0)	1	2.3	0.50

After EDC treatment we next checked the effect of citrate (pH 3.0) on the modified amino acid residues. The results show that citrate treatment does not affect the modified carboxylic groups since electron donation by Mn(II) is the same in EDC-treated PSII(−Mn) membranes before or after incubation in citrate buffer (Fig. 3, compare open triangles with closed circles).

We finally studied the effect of EDC on iron-blocked PSII (−Mn) membranes. After incubating PSII(−Mn) membranes under weak light with iron to block the HA_Z site [34], we washed the membranes, incubated them with 22 mM EDC under the same conditions as before, pelleted membranes, washed and then suspended them in citrate buffer (pH 3.0) to extract the blocking iron cations. After a 2-min incubation, the samples were washed twice in buffer A, and Mn(II) donation was measured (Fig. 4). The data show that the citrate-treated, EDC-modified PSII(−Mn,+Fe) membranes have only one donation site instead of two found in the EDC-treated PSII (−Mn) samples (see Fig. 3). Its K_d (2.3 μM) corresponds to the K_d value of the HA_Z site in EDC-treated PSII(−Mn) membranes (1.52 μM). Thus, EDC-treatment of Fe-blocked PSII(−Mn) membranes is not accompanied by the appearance of the LA_Z site. These results indicate that a carboxylic residue(s) at the HA_Z site cannot be modified by EDC in blocked membranes since it is protected by bound iron cation(s). Thus, the protected carboxylic residue associated with the HA_Z site participates in the ligation of the iron cation blocking the HA_Z site.
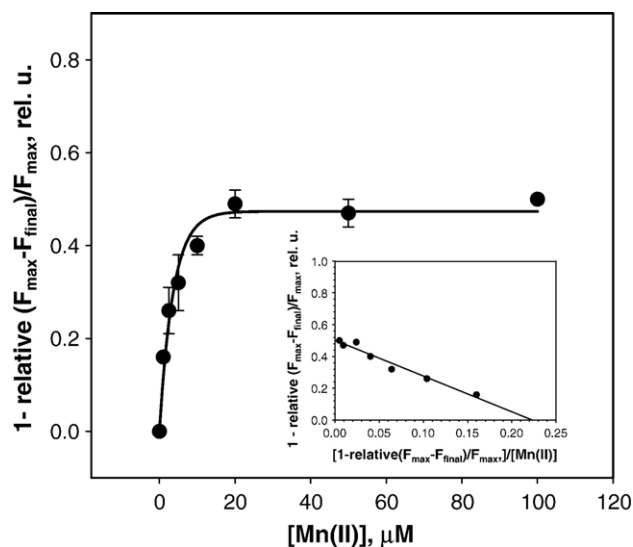

Fig. 4. Concentration dependence of electron donation by Mn(II) to $Y_Z^{\bullet}$ in PSII (−Mn) membranes that were Fe-blocked, EDC-modified, and then citrate-treated (solid circles). The data were fitted with one exponential (solid line). PSII (−Mn) membranes (25 μg Chl/mL) were incubated with 15 μM Fe(II) under weak light (4 μE/m^2 s) for 1.5 min to block the HA_Z, Mn-binding site. After removing excess Fe(II) cations by centrifugation, PSII(−Mn,+Fe) membranes (0.25 mg Chl/mL) were incubated for 1h in buffer A at room temperature in dark with 22 mM EDC. Thereafter, the samples (25 μg Chl/mL) were incubated in citrate buffer C for 1.5–2 min to extract the iron cations blocking the HA_Z site (see Fig. 1 and Table 1 for details). After the samples were washed twice with buffer A, the concentration dependence of electron donation by Mn(II) cations was measured. For other details, see the legend to Fig. 2. Insert: Scatchard plot of the above data. The *x*- and *y*-intercepts were used to estimate the binding affinity, K_d, for Mn(II) at the HA_Z site and the relative amount of binding sites.

4. Discussion

4.1. Extraction of blocking iron cations

In this study, we found that citric acid at pH 3.0 with a stability constant, $K_{st}=11.85$, for the citrate/Fe(III) complex quickly and fully extracts the iron cations blocking the HA_Z, Mn-binding site in PSII(−Mn) membranes. However, the same citrate treatment of intact PSII only extracts calcium cation from the OEC, but not manganese [50]. The different effects of citrate on the Mn(III or IV) cations and Fe(III) cations, ligated by the carboxylic group(s) at the HA_Z, site can be explained by (a) a different value of K_{st} for complexes of Mn(III or IV) and Fe(III) with carboxylic amino acids or (b) the presence of the Mn-stabilizing protein (the 33-kDa extrinsic protein) on the donor side of intact PSII samples [50], but the absence of this protein in Mn-depleted, Fe-blocked samples. One or both of these factors could determine the different resistances of manganese and iron cations to the action of citrate. Indeed, in intact PSII membranes, Mn(III) and especially Mn(IV) located at the HA_Z site, can be bound more tightly than Fe(III) at the same site in Mn-depleted PSII membranes. Also, in intact PS(II) membranes, the Mn cluster is protected by the extrinsic, 33-kDa protein, which is not extracted during citrate treatment, in contrast to the 16 and 23 kDa extrinsic proteins (the latter, however, rebind after dilution of the citrate buffer with MES buffer used to readjust the pH to 6.5) [53] The role of the extrinsic, 33-kDa protein in stabilizing the Mn cluster is well known [54].

The efficiency of Fe(III) extraction by citrate resembles that of Ca^{2+} extraction [50], and one could suggest that iron binds to the Ca-binding site. However, the following results indicate that this is not the case. First, the interaction of Mn(II) and Fe(II) with the HA_Z site is mutually exclusive [29]. Since the Mn(II) and Mn(III) are bound at the same site as determined by previous mutagenesis and crystal structure studies (at least the same amino acid, D1-Asp170, participates in the ligation of Mn at "both" sites) [1,2,30] and since Fe(III) is bound at the Mn(II)/Mn(III) binding site (this study), we can conclude that Fe(II) and Fe(III) are also bound at the same site, which cannot be the Ca-binding site. Second, we have shown that Ca^{2+} inhibits the interaction of Fe(II) cations with the HA_Z Mn-binding site (i.e., the donation of electrons by Fe(II) to $Y_Z^{\bullet}$) to a very limited extent. The decrease in donation was 25% at 5 μM Fe(II) and 50 mM Ca^{2+}. The inhibition of Fe blocking, a more complex process (requiring the oxidation of 4−5 iron cations [43]) than oxidation of the first Fe(II) cation, was a little larger (a 42% decrease) but not 100%, and it was observed at the same very high concentration ratio of Ca^{2+} to Fe(II) (unpublished data). So, the weak effect of Ca^{2+} and the very large ratio between concentrations of Ca^{2+} and Fe(II) required also suggests that the Fe-binding site is not the Ca-binding site.

The decay kinetics of the fluorescence from PSII(−Mn,+Fe) samples treated with citrate at pH 6.5 clearly show that some of the centers still contain iron cations. Increasing the citrate concentration (30 mM, Table 1) had little effect on the extraction efficiency. Thus, the efficiency of extraction is a function of two parameters, pH and the chelating properties of

citrate; only the efficiency of the chelator at pH 6.5 limits iron extraction. Indeed, EDTA, which binds the Fe(III) with $K_{st} = 25.7$, does not extract the blocking iron after treatment for 1 min at pH 6.5 [27] and in fact requires more time to extract iron (data not presented). The important role of low pH for iron extraction indicates that carboxylic groups for the most part form the coordination sphere around the iron cluster.

4.2. EDC-treatment

To study the role of the carboxylic residue in the ligation of iron cation(s) blocking the HA_Z site, we used iron cations to protect the carboxylic ligand against the action of the chemical modifier EDC. Similar approaches were used by Preston and Seibert [25] and Magnuson and Andreasson [20] but with other metal cations. Preston and Seibert found that only Mn(II) cations (5 mM) could protect the HA_Z site from EDC modification, whereas Ca, Ba, Zn, Cu, Mg and Co were less effective then Mn (II). They prevented inhibition of only 50% of the donation centers as determined by the DPC-inhibition assay. Magnuson and Andreasson [20] compared the effect of chemical modification on intact PSII membranes (where the carboxylic ligands were protected by the tetrameric Mn cluster) and Mn-depleted membranes. Modification of carboxylic acid residues in Mn-depleted PSII membranes increased the K_M for Mn(II) oxidation about 5-fold compared to samples modified in the presence of the Mn cluster. Although in this study, the functional carboxylic groups at the HA_Z site were protected not only by the presence of Mn ions but also by the extrinsic proteins. Our data demonstrate that the protective action of bound iron cations against EDC modification is also very high and equal almost to 100%. Thus, our results again show the high specificity of Fe(II) cation interaction with the HA_Z, Mn-binding site.

The treatment of Mn-depleted PSII membranes with EDC is accompanied by the appearance of the LA_Z site (Table 2). The LA_Z site can bind the Mn(II) cation that is oxidized by the Y_Z radical. However, the nature of the LA_Z site is unclear—is it a modified HA_Z site that has a higher K_d due to the modification of one or several carboxylic ligands, or is it a new, "uncovered" Mn-binding site? From an examination of the recent PSII structure [1,2], the HA_Z site could be the site binding the Mn4 [1] (Mn55 according to the numbering in [2]) ion since Asp-170 is the ligand for this cation [13,30,35–38]. If this is true, besides D1-Asp170, the HA_Z site could also be composed of the carboxylic amino acid residue, D1-Glu333. Therefore, if these carboxyl residues are modified at different rates (if Asp-170 is modified faster than Glu-333 for example), the LA_Z site is a modified HA_Z site. On the other hand, if the reaction rates of these residues with EDC are similar, there would be no ligands to bind cations at the HA_Z site, assuming that modified carboxylic groups cannot bind metal cations. In this case, the LA_Z site could be formed by ligands participating in the coordination of other Mn cations and could be considered an uncovered Mn-donation site.

Mutagenesis studies found that in mutants with a substituted D1-Asp170 residue, the K_M for Mn binding increased to 20–60 μM from 1 μM, characteristic of WT material [30]. Besides

this aspartate, a number of mutations have also been constructed at D1-Glu333 [37,55]. Of all these mutants, only D1-Glu333Gln was weakly photoautotrophic [37]. It was proposed that mutations at this site affect the assembly or stability of the Mn cluster and that this glutamic acid participates in $(Mn)_4/Ca$ cluster ligation [31,37]. However, mutations at Glu333 do not affect the ability of the HA_Z site to ligate the first Mn ion that is ultimately incorporated into the $(Mn)_4/Ca$ cluster during the process of photoactivation [37,55]. But, as suggested by Burnap [56], this Mn might be ligated by Glu333 after its oxidation at D1-Asp170 (due to an increase in the ligand field stabilization energy of $X-Mn^{3+}$). Therefore, these results in addition to our observations show that EDC-induced inhibition of the HA_Z site occurs as the result of modifying only one aspartate residue. Thus, the LA_Z site is most likely a newly uncovered site. Finally, our results also demonstrate that the carboxylic residue participating in the coordination of the iron cation(s) blocking the HA_Z site is D1-Asp170.

Acknowledgements

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