Effects of Carboxyl Amino Acid Modification on the Properties of the High-Affinity, Manganese-Binding Site in Photosystem II†

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ABSTRACT: Our previous work using the "diphenylcarbazide (DPC)-inhibition assay" has identified four amino acid (two carboxyls and two histidyls) ligands to four Mn^{2+} bound with high affinity on Tris-washed photosystem II (PSII) membrane fragments [Preston and Seibert (1991) *Biochemistry 30*, 9615−9624, 9625−9633]. One of the ligands binds a photooxidizable Mn, specifically, and the others bind either nonphotooxidizable Mn^{2+}, Zn^{2+}, or Co^{2+} [Ghirardi et al. (1996) *Biochemistry 35*, 1820−1828]. The current paper shows the following: (a) the high-affinity photooxidizable Mn, which donates to the oxidized primary PSII donor ($Y_Z^{\bullet}$), is bound to a carboxyl residue with a $K_M = 1.5\ \mu M$ or $K_d = 0.94\ \mu M$ in the absence of DPC, and a $K_i = 1.3\ \mu M$ in the presence of DPC (both steady-state and flash approaches were used); (b) if this carboxyl is chemically modified using 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC), Mn^{2+} is photooxidized at a lower affinity ($K_d = 25\ \mu M$) site that does not involve carboxyl ligands; (c) low-affinity Mn is photooxidized (possibly by $Y_D^{\bullet}$, the oxidized form of the alternative PSII donor) with a $K_M = 220\ \mu M$ at a completely different site that also requires a carboxyl ligand; (d) photooxidation of high-affinity DPC by $Y_Z^{\bullet}$ with a K_M of 40−42 μM or K_d of 49−58 μM occurs at a site that does not require carboxyl residues; (e) photooxidation of low-affinity DPC with a $K_M = 1200\ \mu M$ occurs at a site (possibly near Y_D) that is not affected by carboxyl modification with EDC. Due to the similarities between the binding of the high-affinity photooxidizable Mn to EDC-treated membranes and to PSII complexes from Asp170D1 mutants [Nixon and Diner (1992) *Biochemistry 31*, 942−948], we identify its carboxyl residue ligand as Asp170 on D1, one of the reaction-center proteins. The second carboxyl ligand identified using the DPC-inhibition assay binds Mn (but not a photooxidizable one), Zn, or Co ions. At least one of the two histidyl ligands (either His337 on D1 or another unidentified histidyl) that bind nonphotooxidizable, high-affinity Mn^{2+} also binds Zn^{2+} and Co^{2+}.

The tetrameric manganese cluster, a part of the photosystem II (PSII)[1] water-oxidizing complex, has two primary functions: it helps accumulate four positive charges prior to the four-electron oxidation of water and provides binding sites for two water molecules (*1*, *2*). The minimal PSII O_2-evolving unit is comprised of the D1/D2/cyt b_{559} reaction center complex, the 43 and 47 kDa intrinsic proteins, the extrinsic 33 kDa protein, and several low molecular weight polypeptides (*1*, *3*). While it is not totally clear which polypeptides provide ligands to the four Mn ions that form the cluster, early experimental evidence supports the idea that Mn ligands are localized on either the D1 (*4*) or the D2 (*5*) proteins. However, the involvement of CP47 and the extrinsic 33 kDa protein in either ligating Mn or stabilizing the Mn cluster is also a possibility (see review by *6*). Recent spectroscopic results support the idea that the Mn cluster is asymmetrically situated with respect to tyrosines Y_Z and Y_D (however, see also *7*) only 4.5 Å from the former (*8*) and about 30−40 Å from the latter (*9*). As a consequence, the cluster is probably bound primarily to D1 residues.

Potential amino acid ligands to the Mn cluster have been identified either with systems in which binding of exogenous Mn with high affinity to Tris-washed membranes is prevented by amino acid chemical modification or limited proteolysis (*10*−*16*), or with site-directed mutants (*5*, *17*−*22*). It has not been clear whether the two approaches identify the same set of ligands, or whether the formation of the cluster involves a different set of ligands compared to those that actually bind the photoactivated cluster.

In the past, we have used the "DPC-inhibition assay" to investigate potential ligands to Mn uncovered by Tris-washing PSII membrane fragments. The assay measures Mn^{2+} inhibition of DPC-supported DCIP photoreduction, a phenomenon that is not observed in control PSII membranes containing the Mn cluster. Under our experimental conditions, 50% of the rate of DCIP photoreduction by DPC alone

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[1] Abbreviations: Chl, chlorophyll; CP, chlorophyll-binding protein; cyt b_{559}, cytochrome b_{559}; D1 and D2, major reaction center proteins of photosystem II; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; F_{final}, fluorescence yield detected after decay of the flash-induced F_{max}; F_{max}, maximum fluorescence yield following actinic flash excitation; K_d, dissociation constant of a substrate−enzyme complex; K_i, dissociation constant of an enzyme−inhibitor complex; F_V, variable fluorescence yield; K_M, Michaelis−Menten constant for an enzymatic reaction; MES, 2-(*N*- morpholino)-ethanesulfonic acid; PS, photosystem; Q_A, first plastoquinone acceptor on the reducing side of PSII; Tris, tris(hydroxymethyl)aminomethane; V_{max}, maximum initial rate of an enzymatic reaction; Y_D, tyrosine 160 on the D2 protein; Y_Z, tyrosine 161 on the D1 protein.

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is due to electron transfer through Y_Z, while the other 50% is due to photooxidation of DPC at a low-affinity site (*23*, *24*) possibly near Y_D (*23*). Addition of up to 10 μM $MnCl_2$ inhibits only that 50% associated with Y_Z due to binding of Mn with high affinity at a site near Y_Z. This inhibition does not require turnover of bound Mn (*24*). Our previous analyses using combinations of proteolytic enzymes and amino acid modifiers have shown that Mn inhibition at the high-affinity site can be deconvoluted into four independent components, which we attributed to four different amino acid residues ligating each of four Mn ions (*10*, *13*−*16*). Two of the four ligands are inactivated by the histidyl modifier DEPC, and the other two ligands are inactivated by the carboxyl modifier EDC (*15*, *16*). Recently, we showed that only one of the four bound Mn is photooxidizable by a single flash (referred to hereafter as the photooxidizable Mn) and that its photooxidation is not inhibited by Zn^{2+} nor Co^{2+}. In other words, it is bound by a Mn-specific ligand. The other three bound Mn are not photooxidizable by a single flash and may share ligands with Zn^{2+} and Co^{2+} (*24*). We also suggested that the Mn-specific component of the high-affinity, Mn-binding site identified using the DPC-inhibition assay was the same ligand that binds the photooxidizable Mn involved in the first step of the photoactivation process (*24*).

In this paper, we identify the amino acid that binds the photooxidizable Mn as a carboxyl residue. Given the similarities between the binding affinity of Mn to PSII membranes before and after EDC modification and to PSII complexes before and after site-directed mutagenesis of Asp170D1 (*18*), we propose that this carboxyl ligand is Asp170. If this residue is chemically modified, Mn^{2+} is photooxidized at an alternative lower affinity site not associated with a carboxyl residue. Additionally, low-affinity Mn and high-affinity DPC are photooxidized at sites associated with and not associated with carboxyl residues, respectively. For the first time now, we can provide a correlation between a Mn ligand detected by the DPC-inhibition assay and a specific amino acid on D1 that has been found by site-directed mutagenesis to be essential for ligation of Mn required for forming the tetrameric Mn cluster in PSII. Furthermore, the recent work of Chu et al. (*21*, *22*) has confirmed our original assignment of His337 on D1, another component of the high-affinity site (*14*−*16*), as a possible Mn ligand involved in the water oxidation process.

MATERIALS AND METHODS

Thylakoids and photosystem II-enriched membrane fragments (PSII membranes) were prepared from market spinach as described by Ghirardi et al. (*24*) and resuspended in K4 buffer (20 mM MES, pH 6.5, containing 400 mM sucrose, 15 mM NaCl, and 5 mM $MgCl_2$). The membranes were stored at −80 °C until use. Thawed membranes were Tris-washed to remove the tetrameric Mn cluster and the extrinsic polypeptides (*24*), and treatment of membranes with the carboxyl modifier EDC was done in buffer A (50 mM MES buffer, pH 6.5, containing 400 mM sucrose and 20 mM NaCl) at 20 °C in the dark for 1 h as in Preston and Seibert (*15*). Nucleophiles such as glycine were not included in the modification buffer (*12*) because our previous studies showed that cross-linking of EDC to amino groups had no effect on the high-affinity Mn site (*15*).

Steady-state light-induced DCIP photoreduction rates were measured from initial absorption changes at 600 nm (*15*), and unless otherwise stated, the following reactant concentrations were used: 200 μM DPC, 10 μM $MnCl_2$, and 50 μM DCIP. Flash-induced, variable fluorescence yields were monitored at ≥715 nm as a function of time with a home-built instrument (*24*). Data analysis for both experiments used Data Translation Global Lab® software and a DT2839 A/D board mounted in an ALR 486 PC (*24*). Samples used for fluorescence measurements were dark-adapted and resuspended in buffer A at a final Chl concentration of about 25 μg/mL. DCMU was added to a final concentration of 50 μM. Enzyme kinetic analysis was according to Segel (*25*), and K_i's were obtained from I_{50} measurements (Mn and DPC are noncompetitive). Curve-fitting employed Grafit 3.0 software (*26*).

RESULTS

The carboxyl modifier EDC has been used extensively in biochemical research to selectively investigate the role of aspartate and glutamate amino acid residues on various cell functions (*27*). The selective inactivation of carboxyl residues that contribute ligands to Mn associated with PSII function was accomplished in the past by treating Tris-washed or hydroxylamine-washed PSII membranes with EDC (*12*, *15*, *16*, *28*, *29*). The rebinding of Mn to the membranes was then investigated, and the loss of functional Mn was correlated with the appearance of modifiable carboxyl residues. As mentioned in the introduction, our previous work has shown that binding of ≤4 Mn to Tris-washed PSII membranes can be detected [by ICP measurements (*16*)] under the conditions of the DPC-inhibition assay. Addition of 10 μM Mn^{2+} to these membranes decreases the initial rate of DPC (200 μM)-supported DCIP photoreduction by about 50%, which corresponds to the binding of 4 $Mn^{>+2}$ ions (*10*, *15*, *16*). Treatment of Tris-washed PSII membranes with EDC decreases the initial rate of DPC-supported DCIP reduction by only 25% in the presence of 10 μM Mn^{2+} and thus eliminates the ability of the assay to detect two of the ligands to Mn. This observation led to the conclusion that two of the high-affinity Mn ligands detected by the DPC-inhibition assay are carboxyl residues (*15*).

To further study the effect of EDC on high-affinity Mn binding to PSII, we initially investigated whether the carboxyl modification was specific for Mn or whether it also affected DPC electron donation to PSII. Table 1 shows that 10 mM EDC inhibits light-induced electron transport from DPC (in the absence of Mn) to DCIP to about 66% of the control rate (row 1). The effect of EDC treatment on DCIP photoreduction by $MnCl_2$ (in the absence of DPC), on the other hand, is much greater, and results in a decrease to about 32% of the control rate (row 2). These data are qualitatively similar to those of Tamura et al. (*12*) and suggest that photooxidizable Mn in PSII is bound mostly to carboxyl residues. The preferential effect of EDC treatment on Mn photooxidation (see later for similar flash results) might be explained alternatively by nonspecific cross-linking of PSII proteins, blocking the access of Mn to Y_Z (*15*). However, we find it improbable that such cross-linking would not also prevent access of DPC (a much larger molecule) to Y_Z on PSII. The lack of complete inhibition of Mn photooxidation by 10 mM EDC under the conditions of Table 1 (row 2)

Table 1: Initial Rate of DCIP Photoreduction by Control and EDC-Modified, Tris-Washed PSII Membranes Mediated by Different Combinations of Donors and Inhibitors[a]

electron donor	rate of DCIP photoreduction [μmol of DCIP (mg of Chl)$^{-1}$ h^{-1}] control PSII	EDC-treated PSII	treated/ untreated
200 μM DPC	115.60	76.07	66%
10 μM $MnCl_2$	32.93	10.69	32%
200 μM DPC + 10 μM $MnCl_2$	57.81 (50)	60.67 (80)	na
200 μM DPC + 100 μM $ZnCl_2$	71.27 (62)	61.79 (81)	na
200 μM DPC + 100 μM $CoCl_2$	70.66 (61)	62.10 (82)	na

[a] Rows 1 and 2 show the effect of EDC (10 mM) modification on the rates of DCIP photoreduction using either DPC or Mn as donors to PSII. The relative decrease in the rates between EDC-modified and unmodified samples using either DPC or Mn as donors (column 4) indicates the preferential effect of EDC on Mn binding. Rows 3−5 show the effect of added metals to control or EDC-modified samples on the rate of DCIP photoreduction, using DPC as the electron donor to PSII. The values in parentheses (columns 2 and 3) represent the percentage of the DPC-mediated DCIP photoreduction rates (from row 1) that are *not inhibited* by addition of each metal. Residual rates of 50−62% (seen upon addition of 10 μM $MnCl_2$, 100 μM $ZnCl_2$, or 100 μM $CoCl_2$ to control samples) represent the binding of the full complement of metal ions to PSII; 80−82% (seen upon addition of the same metals to EDC-modified samples) corresponds to the binding of only about half of the ions to PSII.

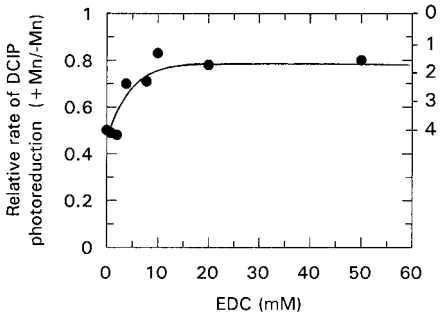

FIGURE 1: Relative initial rate of DCIP photoreduction by 200 μM DPC measured in the presence and absence of added 10 μM $MnCl_2$. The measurements were done with Tris-washed PSII membrane fragments chemically modified with different concentrations of the carboxyl-modifier EDC. The right *y*-axis indicates the number of unmodified components of the high-affinity, Mn-binding site (i.e., available Mn ligands) that correspond to the given relative rates at each EDC concentration, according to Preston and Seibert (*15*, *16*).

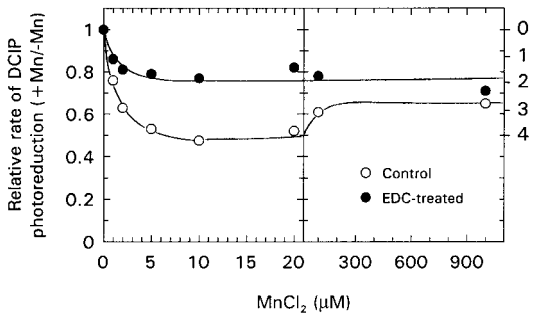

FIGURE 2: Relative rate of DCIP photoreduction by 200 μM DPC as a function of added $MnCl_2$. The measurements were done either with unmodified Tris-washed control PSII membranes (open circles) or with membranes modified with 10 mM EDC (closed circles). The right *y*-axis is as in Figure 1.

may be due to (a) the incomplete modification of high-affinity, Mn-binding carboxyl residues on PSII by this particular concentration of EDC, (b) photooxidation of high-affinity Mn bound to histidyl residues in these samples under continuous illumination *in the absence of DPC*, or (c) selective inactivation of the ligand to high-affinity Mn that is photooxidized by $Y_Z^{\bullet}$ (and that contributes 87% of the steady-state rate of DCIP photoreduction under the assay conditions) but no effect of the treatment on the ligand(s) that bind(s) Mn photooxidized at the low-affinity site (*23*) and contribute(s) the remaining 13% of the steady-state DCIP photoreduction rate (*24*).

The first possibility was investigated by titrating the effect of EDC on Mn binding to PSII using the DPC-inhibition assay to estimate the number of modified high-affinity, Mn-binding sites. Figure 1 shows the rate of DCIP photoreduction by 200 μM DPC in the presence of 10 μM $MnCl_2$, relative to the rate measured in the absence of $MnCl_2$, plotted as a function of EDC concentration in the modification medium. The *y*-axis on the right side of the figure indicates the number of detectable Mn-binding ligands that correspond to the measured relative initial rates as demonstrated by Preston and Seibert (*15*, *16*). It is clear that the loss of Mn-binding ligands reaches a maximum (two of a possible four) after modification with 10 mM EDC since an increase in the EDC concentration up to 50 mM has little if any additional effect. These results demonstrate that the modification of the high-affinity carboxyl ligands binding Mn is complete when 10 mM EDC is used and that the partial inhibition of Mn photooxidation resulting from EDC treatment cannot be explained by incomplete modification of high-affinity, Mn-binding carboxyl residues. Besides invalidating our first hypothesis, this is the reason 10 mM EDC was used in the following work, unless stated differently in the text.

The second hypothesis proposes that incomplete inhibition of Mn photooxidation after EDC modification is due to steady-state photooxidation of Mn bound to histidyls residues in modified samples. Our previous studies examining EDC- and DEPC-modified samples have suggested that neither treatment changes the K_i for Mn inhibition of DPC-supported DCIP photoreduction (*15*, *16*), which indicates that carboxyls and histidyls bind Mn with about the same affinity. However, it has been argued that Mn binding to carboxyl residues is tighter (has a lower K_M) than its binding to histidyl residues in PSII (*28*, *29*). If this were the case, we should also observe changes in the binding of high-affinity Mn to PSII using the DPC-inhibition assay after EDC modification. We have reassessed the binding affinity of Mn to histidyl residues in Tris-washed spinach PSII membranes by estimating the change in K_i for Mn inhibition of DPC photooxidation before and after the membranes are treated with EDC. Figure 2 shows the rate of DCIP photoreduction by 200 μM DPC in the presence of $MnCl_2$ relative to the rate measured in the absence of $MnCl_2$ as a function of added $MnCl_2$. Addition of $MnCl_2$ results in a decrease of the rate of DCIP photoreduction to a minimum of about 50% at 10 μM $MnCl_2$ in control membranes (open circles), but only to about 78% in EDC-treated membranes (closed circles). Higher concentrations of added $MnCl_2$ up to 1 mM have little further effect on the rate of DCIP photoreduction in EDC-treated

samples but, as discussed previously (*24*), cause an increase in the relative rate of DCIP photoreduction in control membranes. This latter phenomenon is due to additional photooxidation of Mn^{2+} (when higher concentrations are present) that can occur at the low-affinity site in Tris-washed PSII membranes (*30*) under the DPC-inhibition assay conditions (*31*).

The estimated K_i in Figure 2 for Mn binding at high affinity measured in control membranes was about 1.3 μM, while that in EDC-treated membranes was about 1 μM. Since in EDC-treated membranes only histidyls are expected to bind the remaining two components of the high-affinity, Mn-binding site, these results show that Mn inhibits DPC photooxidation with about the same affinity when bound only to histidyl or to both histidyl and carboxyl residues on PSII membranes. This confirms and extends our previous studies (*15*, *16*, *31*), but still conflicts with the observations of others suggesting lower binding affinity of Mn to histidyls as mentioned above. This discrepancy may arise because of the different nature of our assay for Mn binding compared to other groups. The DPC-inhibition assay that we use requires binding of Mn in the presence of DPC and does not require steady-state photooxidation of Mn (*24*, *31*), while Blubaugh and Cheniae (*28*) and Magnuson and Andreasson (*29*) monitored Mn-binding affinities by assaying for Mn-supported DCIP photoreduction in the absence of DPC. Thus, it is possible that Mn bound to histidyl residues is only photooxidized in the absence of DPC which would confirm the second hypothesis. This issue will be examined in detail later in the paper by means of single-turnover flash experiments.

Finally, the incomplete inhibition of Mn photooxidation by EDC modification might be explained by electron donation from Mn^{2+} bound to non-carboxyl residues at the low-affinity site (our third hypothesis). However, the data in Figure 2 show that only carboxyl (and not histidyl or other) ligands can facilitate electron donation by Mn^{2+} to the low-affinity site, which is inconsistent with the third hypothesis.

Besides differences in their ability to bind Mn that is photooxidized even in the presence of DPC, ligands to high-affinity Mn as detected by the DPC-inhibition assay have different affinities for other metal ions (*24*). The ligand to the photooxidizable Mn is specific for Mn. The other three ligands to Mn that is not photooxidized by a single flash may also bind other metals such as zinc and cobalt. Table 1 shows that the EDC modification partially removes sites that bind manganese, zinc, and cobalt involved in DPC inhibition, indicating that the second high-affinity, Mn-binding carboxyl ligand must also bind these other metals.

The use of the DPC-inhibition assay to identify potential ligands to the physiological Mn cluster has been criticized on the basis of its complexity (*32*). Indeed, the assay depends on the integrity of (a) light absorption by antenna Chl and charge separation function of PSII, (b) DCIP reduction on the reducing side of PSII, (c) oxidation of DPC by $Y_Z^{\bullet}$ and by $Y_D^{\bullet}$ on the oxidizing side of PSII, and (d) binding of Mn on the oxidizing side of PSII. The use of amino acid modifiers may affect any of these functions and could potentially lead to a misinterpretation of the results. Moreover, the existence of (a) high- and low-affinity binding sites for both DPC and Mn at PSII (*24*, *30*, *31*), where, respectively, $Y_Z^{\bullet}$ (*18*, *30*, *33*, *34*) and possibly $Y_D^{\bullet}$ (*23*) are reduced, and (b) several types of ligands for high-affinity Mn (*24*) makes it a challenge to assess the specific effect of amino acid modifiers on high-affinity DPC and/or Mn binding using steady-state techniques. To circumvent these issues, we have used flash-probe fluorescence decay measurements to study the specific effects of EDC on ligands to high-affinity photooxidizable DPC and Mn.

Flash-induced fluorescence decay measurements monitor charge recombination between $Y_Z^{\bullet}$ and Q_A^- generated by an actinic flash (see *3* for a review). In Tris-washed, DCMU-treated PSII membranes, an actinic flash generates the high fluorescent state $Y_Z^{\bullet}Q_A^-$ (F_{max}) in about 20−40 μs (*32*). These species recombine in 15−100 ms (*17*−*20*, *35*), giving rise to a low fluorescent state, F_{final} (*3*, *36*, *37*). Addition of increasing concentrations of exogenous PSII donors results in a rise in the F_{final} fluorescence level (*24*) due to accumulation of Q_A^- (*18*). It is possible to estimate the binding affinity of a donor to $Y_Z^{\bullet}$ by titrating the amount of accumulated Q_A^- (relative to that measured in the absence of exogenous donors) as a function of the concentration of added donor (*18*). We used F_{final}/F_{max} as a measure of accumulated Q_A^-. To correct for the nondecaying F_{final} that is present even in the total absence of electron donors to PSII in Mn-depleted samples (*38*), and to normalize the above ratio to F_{final}/F_{max} measured in the absence of added donor, we (a) measured the decrease in the $(F_{max} - F_{final})/F_{max}$ ratio (which represents the inverse of the accumulated Q_A^-) at each donor concentration, (b) divided it by the same ratio obtained in the absence of added donor (which represents the maximum amount of accumulated Q_A^-), and (c) subtracted this ratio from 1 (to obtain the percentage of accumulated Q_A^- as a function of donor concentration). This expression is represented by the equation [1 − relative (F_{final}/F_{max})]. This analysis assumes that electron donation by the exogenous donor is faster than charge recombination, and thus that the amount of accumulated Q_A^- depends only on the binding affinity of the donor (*18*, *33*; however, see *34*). The relationship between fluorescence yield and Q_A^- concentration is not linear, unless the fluorescence measurements are done in the absence of divalent cations (*39*). To extrapolate the results from our fluorescence measurements into accumulation of Q_A^-, our experiments were done in a medium devoid of cations as well. The data were analyzed as Scatchard plots, where [1 − relative (F_{final}/F_{max})] was plotted as a function of [1 − relative (F_{final}/F_{max})]/[donor]. The slope of the line in the Scatchard plot is an estimate of the apparent binding affinity of the donor (K_d), and the *y*-intercept determines the relative amount of donor-binding PSII centers.

Since DCMU is required to block electron transfer beyond Q_A^- in the above-described experiments, we first examined the effect of EDC modification on DCMU binding to thylakoid membranes [the lumenal side of the membrane in thylakoids is sequestered whereas it is not in PSII membrane fragments (*40*)]. EDC is not expected to affect DCMU binding because carboxyl residues are not a part of the DCMU-binding site (*41*), but longer range affects might be possible. Figure 3 shows the rates of DCIP photoreduction using H_2O as the donor in control (open circles) and EDC-modified (closed circles) thylakoids as a function of DCMU added to the assay medium. The inset is a Dixon plot (1/

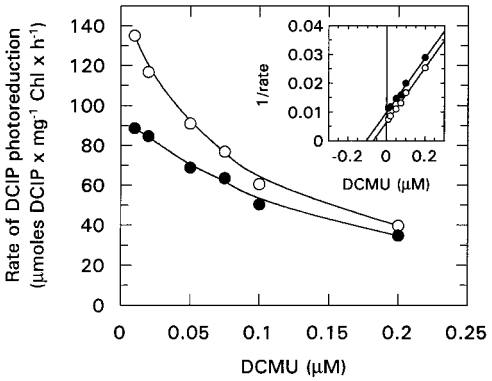

FIGURE 3: Rate of water-supported DCIP photoreduction as a function of added DCMU, measured with either unmodified control thylakoid membranes (open circles) or thylakoid membranes modified with 10 mM EDC (closed circles). Inset: Dixon plot of the data. The *x*-intercepts of the two straight lines indicate that the EDC treatment does not significantly change the binding affinity of the membranes for DCMU.

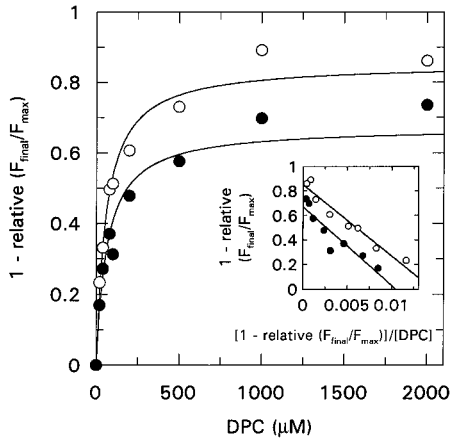

FIGURE 4: The [1 − relative (F_{final}/F_{max})] ratio as a function of added DPC using unmodified control (open circles) and 10 mM EDC-modified (closed circles), Tris-washed PSII membranes. [1 − relative (F_{final}/F_{max})] is a measure of the number of PSII centers undergoing an initial light-induced charge separation reaction that do not recombine. Inset: Scatchard plot of the data. The *y*-intercept is an estimate of the number of PSII centers undergoing a charge recombination reaction following an actinic flash; the slope of the line is an estimate of the binding affinity (K_d) of DPC to the membranes.

rate versus inhibitor concentration) showing that EDC causes a trivial increase in the K_i of DCMU (from 0.07 to 0.10 μM), as seen at the *x*-axis intercept of the two lines. As a result, the rate of DCIP photoreduction measured in the presence of 50 μM DCMU (the concentration used in the flash-induced fluorescence decay experiments) will be only 1% of V_{max} in control samples and 2% of V_{max} in EDC-treated samples, both of which are negligible in the following experiments as determined by applying the Michaelis−Menten equation to both sets of data. Tamura et al. (*12*) have stated that the I_{50} for DCMU inhibition of DCIP reduction does not change upon EDC treatment of PSII membranes, but the design of their experiment would not have detected offsetting effects of EDC on both sides of the membrane.

The effect of EDC treatment on DPC donation to PSII with high affinity is seen in Figure 4. Here, [1 − relative (F_{final}/F_{max})] values measured with control (open circles) and 10 mM EDC-modified (closed circles), Tris-washed PSII membranes are shown as a function of added DPC. The

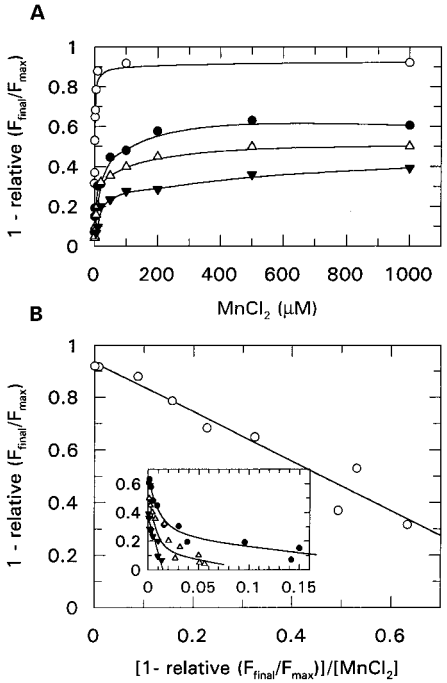

FIGURE 5: (A) The [1 − relative (F_{final}/F_{max})] ratio as a function of added $MnCl_2$ using unmodified control Tris-washed PSII membranes (open circles) and similar membranes modified with 7.8 mM (closed circles), 20 mM (open triangles), or 50 mM (closed triangles) EDC. [1 − relative (F_{final}/F_{max})] is a measure of the number of PSII centers undergoing an initial light-induced charge separation reaction that do not recombine. (B) Scatchard plot of the data shown in (A). The *y*-intercept is an estimate of the number of PSII centers undergoing a charge recombination reaction following an actinic flash; the slope of the line is an estimate of the binding affinity (K_d) of Mn to the membranes. The data for the EDC-treated samples are shown in the inset and are consistent with two components exhibiting separate binding affinities (see Table 2).

Scatchard plots in the inset show that the data are consistent with only one photooxidation site for DPC in either control or EDC-treated membranes. The slope of the lines in the inset is an estimate of the "binding affinity" for DPC at a site oxidizable by $Y_Z^{\bullet}$. The K_d for DPC binding was about 58 μM in control membranes and 64 μM in EDC-treated membranes. These K_d values are close to the K_M value in the presence of DCIP for steady-state DPC photooxidation of 42 μM reported by Blubaugh and Cheniae (*30*) and a K_M of 40 μM reported in our previous paper (*24*). The *y*-intercept determines the relative number of centers that photooxidize DPC. Our results indicate that the EDC treatment inactivates about 21% of the high-affinity, DPC-photooxidizing centers but does not change the K_d of the remaining sites for DPC.

A similar experiment was done using $MnCl_2$ as the electron donor. Figure 5A shows [1 − relative (F_{final}/F_{max})] for control Tris-washed PSII membranes and membranes treated with 7.8, 20, or 50 mM EDC. Figure 5B shows the Scatchard plot for control samples; the inset to Figure 5B is an expansion of the figure that shows the data for the three EDC-treated samples. The binding affinity for Mn (K_d), determined from the control slope of the line in Figure 5B, was estimated to be 0.94 μM. This value is close to the K_M of 1.5 μM that we reported earlier for steady-state photooxidation of Mn (*24*) and to the K_i (1.3 μM) seen for Mn binding using the DPC-inhibition assay (Figure 2). Modi-

Table 2: Relative Number of PSII Centers That Bind High-Affinity and Lower-Affinity Mn, and Their Respective Binding Affinities, As Determined by Flash-Probe Fluorescence Decay Measurements[a]

[EDC] (mM)	no. of centers with high affinity Mn ligand	K_d of high affinity Mn ligand (μM)	no. of centers with lower affinity Mn ligand	K_d of lower affinity Mn ligand (μM)
0	0.933	0.94	–	–
0.75	0.901	1.33	–	–
2	0.944	1.31	–	–
3.8	0.300	0.96	0.427	31
7.8	0.214	0.72	0.433	48.5
10	0.224	0.78	0.385	58.5
20	0.100	1.0	0.430	50
50	–	–	0.367	25

[a] Tris-washed PSII membranes were exposed to the indicated concentrations of the carboxyl-modifier EDC.

fication of PSII membranes with increasing concentrations of EDC results in both a progressive decrease in the relative number of centers that bind high-affinity Mn (*y*-intercepts of the curves shown in the inset) and an uncovering of centers that bind photooxidizable Mn with lower affinity (see closed circles and open triangles in the inset). PSII membranes treated with 50 mM EDC show only a lower affinity binding component for Mn with an estimated K_d of 25 μM (closed triangles). The relative number of centers binding either high-affinity or new lower affinity Mn, as well as the K_ds for Mn, was estimated in Table 2 from Scatchard plots similar to the ones shown in the inset of Figure 5. We assumed that each EDC-modified sample contained a mixture of centers binding either high-affinity or lower affinity photooxidizable Mn. It is clear that increasing the concentration of EDC results in progressive elimination of centers that bind high-affinity Mn with a K_d of about 1 μM, indicating that the Mn ligand must be a carboxyl amino acid residue. The number of centers binding a photooxidizable Mn at the new lower affinity Mn site (K_d of 25−59 μM) stays constant at about 40% above 4 mM EDC.

These results clearly indicate that EDC modification does not completely inhibit Mn photooxidation by $Y_Z^\bullet$, and further eliminate the third hypothesis put forward to explain the incomplete inhibition of steady-state Mn photooxidation after EDC modification. The residual photooxidizable Mn detected in EDC-modified membranes must then be bound either to histidyls (our second hypothesis) or to other amino acid residues on PSII. This problem is addressed in our accompanying paper (*42*) where we use double-modification experiments to show that this lower affinity ligand is not a histidyl residue.

DISCUSSION

Our working hypothesis for identifying some of the amino acid residues on PSII proteins that provide ligands for binding the tetrameric Mn cluster involved in the photosynthetic O_2-evolution process has been to assay for "uncovered" amino acid residues that bind exogenous Mn^{2+} after removing the functional Mn cluster. We have found that Tris-washed PSII membranes (*16*) and isolated PSII D1/D2 reaction centers (*10*) have at least four residues available for binding high-affinity Mn as detected by the DPC-inhibition assay. This assay monitors the inhibition by exogenous Mn^{2+} of steady-state DCIP photoreduction resulting from DPC donation to $Y_Z^\bullet$. The assay works because DPC and Mn^{2+} do not donate electrons to $Y_Z^\bullet$ in the presence of the intact Mn cluster, but can do so in the absence of the cluster (*43*). If Mn^{2+} is present along with DPC, the ion prevents DPC donation to $Y_Z^\bullet$ and, as a consequence, decreases the apparent initial rate of DCIP photoreduction, leading to the results in Figure 2. Possible assay artifacts due to back-reactions resulting from the reoxidation of DCIP by PSII have been eliminated based on kinetic measurements (*15*). Furthermore, the possibility that the ligands detected by the DPC-inhibition assay are actually high-affinity Ca^{2+} (*44*) or Mg^{2+} (*45*) sites that bind Mn (or vice versa) under our conditions is unlikely based on the lack of inhibition of DPC photooxidation by $CaCl_2$ or $MgCl_2$ (*43*) and on ligand-modification protection studies using Mn compared to other divalent cations (*15*).

Different combinations of proteolytic enzymes and amino acid modifiers decrease the inhibitory effect of Mn^{2+}, as measured by the DPC-inhibition assay, in four discrete steps each of which we have interpreted as due to the binding of a Mn ion to a different residue on the D1 and D2 proteins near Y_Z. Two of these residues are carboxyls, and two are histidyls (*15*, *16*). However, only one of the four bound Mn is photooxidizable by a single flash in the presence of DPC (*24*). The ligand to this particular Mn is specific for Mn, and binds neither added Zn^{2+} nor Co^{2+}. The other three ligands bind Mn and may also bind Zn^{2+} or Co^{2+}. None of these three Mn ions is photooxidizable under the above conditions. In the current study, we have used both steady-state and single-turnover flash experiments in combination with treatment using the carboxyl modifier EDC to investigate the nature of the ligand to the photooxidizable Mn.

Both steady-state (Table 1) and flash (Figure 4) experiments show that chemical modification of carboxyl residues on PSII membranes has little effect on the photooxidation of high-affinity DPC (i.e., that photooxidized by $Y_Z^\bullet$). The K_M for steady-state DPC photooxidation by PSII is about 40−42 μM (*24*, *30*) and is consistent with a K_d of 58 μM for DPC photooxidation as determined by flash-fluorescence in unmodified membranes. Neither the K_d nor the relative number of photooxidizable centers changes significantly when the membranes are modified by EDC, and these results demonstrate that carboxyl [and, as we shall see in Ghirardi et al. (*42*) histidyl] residues on PSII membranes are not involved in the interaction between DPC and $Y_Z^\bullet$. Little is known about the residues that are involved in the interaction of PSII with low-affinity DPC, but that DPC is photooxidized with a K_M of 1200−2000 μM (*24*, *30*).

Carboxyl modification, on the other hand, greatly decreases the binding of photooxidizable Mn to the PSII membrane in the absence of DPC (Table 1, Table 2, *12*), demonstrating that the ligand to the photooxidizable Mn in PSII is a carboxyl residue. We showed that the residual noninhibited Mn photooxidation in EDC-modified membranes was not due to incomplete modification of high-affinity carboxyl ligands to Mn (Figure 1) nor due to Mn bound to the low-affinity site (Figure 2). The remaining possibility was that the residual Mn photooxidation was due to photooxidation of high-affinity Mn bound to a histidyl residue(s).

Studies done by Blubaugh and Cheniae (*28*) and Magnuson and Andreasson (29), who calculated binding affinities for Mn in EDC-treated membranes using DCIP photoreduc-

Table 3: Summary of Results About the Identity of Amino Acid Residues That Are Involved in Mn Binding and in DPC Photooxidation, and the Affinities (in Parentheses) for High- and Low-Affinity Photooxidation Sites on PSII Membranes As Determined by Previous Work (*24*, *30*), the Accompanying Paper (*42*), and the Current Study

affinity site	assay	Mn	DPC
high[a]	DCIP photoreduction[b]	one carboxyl, Asp170 on D1, binding a photooxidizable Mn ($K_M = 1.5\ \mu M$)	not a carboxyl ($K_M = 40-42\ \mu M$)
	DPC-inhibition assay[c]	two histidyls, including His337 on D1, and two carboxyls, including Asp170 on D1, binding a photooxidizable Mn ($K_i = 1.3\ \mu M$)	N/A
	flash[d]	one carboxyl, Asp170 on D1, binding a photooxidizable Mn ($K_d = 0.62-0.94\ \mu M$); EDC modification "uncovers" a new lower affinity ligand that is neither a carboxyl nor a histidine[e] ($K_d = 25-59\ \mu M$)	neither a carboxyl nor a histidyl[e] ($K_d = 49-58\ \mu M$)
low[f]	DCIP photoreduction[b]	ligand not examined ($K_M = 225\ \mu M$)	residues involved not examined ($K_M = 1200\ \mu M$)
	DPC-inhibition assay[c]	only Mn bound to a carboxyl residue is photooxidizable	N/A

[a] High-affinity site is associated with Y_Z. [b] Assay measures the initial rate of DCIP photoreduction with Tris-washed PSII membranes using Mn^{2+} or DPC as the PSII donor. The donor affinity is measured as a K_M. [c] Assay measures the initial rate of DCIP photoreduction with Tris-washed PSII membranes using 200 μM DPC as the PSII donor. In the presence Mn^{2+}, DPC donation is inhibited by Mn^{2+} and the affinity of Mn^{2+} is measured as a K_i. [d] Assay measures the amount of charge recombination between $Y_Z^{\bullet}$ and Q_A^- resulting from a single-turnover flash in Tris-washed PSII membranes using Mn^{2+} or DPC as the donor. The donor affinity is measured as a K_d. [e] See the accompanying paper (*42*) for data to support this conclusion. [f] Low-affinity site may be associated with Y_D.

tion as the assay (but in the absence of DPC), showed that the K_M for Mn binding to EDC-treated membranes (presumably containing only histidyl ligands to Mn as judged by the DPC-inhibition assay, Figure 2) is 5−30 times higher than that measured with untreated membranes at the same pH. We tested for the possibility of photooxidizable Mn bound to histidyl residues in the absence of DPC by means of single-turnover flash experiments using control and EDC-modified membranes. Single-turnover experiments, of course, allow us to selectively examine the ligand to the Mn that is photooxidized by $Y_Z^{\bullet}$. Our results indicate that this Mn is bound to a carboxyl residue and that its binding is eliminated by EDC modification (Figure 5). However, EDC modification uncovers a lower affinity ($K_d = 25\ \mu M$) binding site for photooxidizable Mn in about 40% of the centers (Figure 5, Table 2). This uncovered site is not the normal low-affinity Mn site because Mn bound to the low-affinity site does not donate electrons to $Y_Z^{\bullet}$, and thus cannot be detected by flash-fluorescence techniques. The identity of the uncovered ligand to Mn after EDC modification is not known, but as we shall see in the accompanying paper (*42*), it is not a histidine. Table 3 summarizes all the above results.

We have shown previously (*24*) that photooxidation of Mn by a single flash is not inhibited by addition of zinc or cobalt. We now have definitive evidence that the ligand to this photooxidizable Mn (in the presence or absence of DPC) is a carboxyl residue. Nixon and Diner (*18*) examined the effects of site-directed mutagenesis of Asp170D1 in *Synechocystis* 6806 on the binding of the photooxidizable Mn and estimated binding affinities from a titration of the flash-induced absorbance changes in PSII core complexes at 325 nm, corresponding to charge recombination between $Y_Z^{\bullet}$ and Q_A^- (see also *17* and *46*). The amount of accumulated Q_A^- was estimated by the ratio of the absorbance change at 2 s after a flash to that at 0.5 ms. They reported that the K_M for Mn binding in control membranes was 1 μM, and this increased to between 20 and 60 μM in mutant material. From correlations of the similarities in K_d's in the current study before and after EDC modification (Table 2, Figure 5) and those reported by Nixon and Diner (*18*) for K_M's before and after site-directed mutagenesis, we conclude that the carboxyl residue associated with the high-affinity Mn detected by the flash experiments and one of the two carboxyl residues that bind high-affinity Mn detected by the DPC-inhibition assay is Asp170 on D1. This residue photoligates the first Mn required for the photoactivation of the Mn cluster (*18*). The nature of the ligand to the lower affinity photooxidizable Mn uncovered by the EDC treatment as mentioned above is not known, but Mn bound to that residue is not photooxidized by a single flash if Asp170 is present. However, if Asp170 is modified, either by site-directed mutagenesis or by EDC treatment, Mn bound to this secondary residue becomes accessible to $Y_Z^{\bullet}$, and it is oxidized by a single flash, albeit with a lower apparent binding affinity, in 40% of the centers. The chemical modifier EDC is also a protein cross-linker, and its effect on Mn photooxidation could be attributed to this property. However, given the arguments presented under Results and similarities between our results and those from Diner's laboratory (*18*) using site-directed mutants, we must conclude that EDC inhibition of Mn binding to PSII is indeed due to modification of a specific Mn ligand.

The second carboxyl ligand in Tris-washed membranes binds a high-affinity Mn that is not photooxidized under conditions of the DPC-inhibition assay. However, given the partial inactivation of Zn^{2+} and Co^{2+} binding to PSII by EDC seen in Table 1, and the fact that the binding of Mn^{2+} is mixed with respect to the binding of Zn^{2+} and Co^{2+} (*24*), this residue may also bind Zn^{2+} or Co^{2+}. We do not know the identity of this residue, but based on the literature, it could be Glu69 on D2 (*5*), other carboxyls on D2 or other PSII reaction center proteins, or Glu333 on D1 (*21*). Asp342 on D1 (*21*, *22*) is not a candidate for the second high-affinity Mn ligand detected by the DPC-inhibition assay because our previous carboxypeptidase A/chemical modifier experiments have eliminated the possibility of a carboxyl high-affinity ligand beyond Arg334 on D1 (*16*). Extending our reasoning to the two histidyl residues that bind high-affinity Mn as detected by the DPC-inhibition assay, we conclude that at least one may bind zinc and cobalt as well, to partially account for the remainder of the binding components for these metals that were not inactivated by EDC modification. The identity of one of these histidyl ligands is His337 on D1 (*14*, *16*). Recent site-directed mutagenesis studies from Professor Richard Debus' laboratory have confirmed the possible involvement of His337 in binding Mn (*21*, *22*) in contrast to an early report (*47*).

In summary, we have presented evidence in this paper that correlates one of four Mn ligands detected by the DPC-inhibition assay with Asp170 on the D1 protein and have previously identified a second with His337 on D1. While this evidence confirms the relevance of the DPC-inhibition assay for detecting Mn-binding ligands of physiological importance, it is still not clear how all of these Mn ligands are related to the physiological process of photoligation and photoactivation of the tetrameric Mn cluster. In the following paper, we will present additional results on the effects of histidyl modification of high-affinity, Mn-binding ligands.

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