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The Carboxyl Modifier 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) Inhibits Half of the High-Affinity Mn-Binding Site in Photosystem II Membrane Fragments†

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ABSTRACT: The diphenylcarbazide(DPC)/Mn^{2+} assay [Hsu, B.-D., Lee, J.-Y., & Pan, R.-L. (1987) *Biochim. Biophys. Acta 890*, 89-96] was used to assess the amount of the high-affinity Mn-binding site in manganese-depleted photosystem II (PS II) membrane fragments from spinach and *Scenedesmus obliquus*. The assay mechanism at high DPC concentration was shown to involve noncompetitive inhibition of only half of the control level of DPC donation to PS II by micromolar concentrations of Mn at pH 6.5 (i.e., one of two DPC donation sites is inhibited). At low DPC concentration both DPC and Mn^{2+} donate to PS II additively. Treatment with the carboxyl amino acid modifier 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) inhibited half of the high-affinity Mn-binding site in spinach and *Scenedesmus* WT PS II membranes and all of the available site in *Scenedesmus* LF-1 mutant PS II membranes. A similar EDC concentration dependence was observed in all cases. Addition of 2 mM $MnCl_2$ to the 10 mM EDC modification buffer provided complete protection for the Mn-binding site from modification. This protection was specific for Mn^{2+}; six other divalent cations were ineffective. We conclude that EDC modifies that half of the high-affinity Mn-binding site that is insensitive to the histidine modifier diethyl pyrocarbonate (DEPC) [Seibert, M., Tamura, N., & Inoue, Y. (1989) *Biochim. Biophys. Acta 974*, 185-191] and directly affects ligands that bind Mn. The effects of EDC and DEPC that influence the high-affinity site are mutually exclusive and are specific to the lumenal side of the PS II membrane. Removal of the two more loosely bound of the four functional Mn from PS II membranes uncovers that part of the high-affinity site associated with carboxyl but not histidyl residues. We suggest that carboxyl residues on reaction center proteins are associated with half of the high-affinity Mn-binding site in PS II and are involved along with histidine residues in binding Mn functional in the O_2-evolving process.

The O_2-evolving system of photosystem II (PS II)[1] is a metalloenzyme containing four Mn per reaction center. These Mn atoms are believed to form a complex that stores the oxidizing equivalents required for the water oxidation process in a concerted fashion and serves as the catalytic site for O_2 evolution [see Amesz (1983), Dismukes (1986), Babcock (1987), and Hansson and Wydrzynski (1990) for reviews]. Considerable effort has been expended on elucidating the nature of this complex, and while significant progress has been

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[1] Abbreviations: Chl, chlorophyll; D, redox-active tyrosine 160 on the D2 reaction center protein; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; LF-1, low fluorescence mutant of *Scenedesmus obliquus*; MES, 4-morpholineethanesulfonic acid; PS II, photosystem II; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type; Z, redox-active tyrosine 161 on the D1 reaction center protein.

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made, its structure is still unknown. Although detailed information about the binding site of the Mn complex is also unknown, there is now considerable evidence that the D1 protein provides at least some of the ligands required to bind Mn (Seibert & Cotton, 1985; Metz et al., 1986; Seibert et al., 1988, 1989; Tamura et al., 1989). Information from EXAFS studies regarding the nature of the ligands to Mn indicates that either O or N atoms are involved (Kirby et al., 1981; Yachandra et al., 1986). These studies and others (Dismukes & Siderer, 1981; Dismukes et al., 1982) indicate that in fact there are two types of ligands to Mn: terminal ligands to proteins and bridging ligands to other Mn atoms. This report will emphasize studies of potential terminal ligands. Proposed terminal ligands to Mn generally include carboxyl ligands, although other ligands such as amides or histidines are not ruled out (Padhye et al., 1986; Coleman & Govindjee, 1987; Dismukes, 1988). Recently Tamura et al. (1989) used the histidine-modifying reagent DEPC to show that a histidine residue(s) on D1 is involved in binding Mn during photoactivation. Furthermore, histidine oxidation may be involved in the S_2 to S_3 transition in Ca^{2+}-depleted PS II (Boussac et al., 1990), and a photooxidizable histidine residue(s) on the donor side of PS II may provide a redox-active ligand for Mn in Tris-treated PS II membranes (Ono & Inoue, 1991).

An assay for a high-affinity Mn-binding site in Mn-depleted PS II membranes was noted by Hsu et al. (1987), who showed that micromolar concentrations of $MnCl_2$ inhibit DPC-supported DCIP photoreduction. While the specific mechanism they proposed for this assay (competitive inhibition; see the Appendix) is not correct, the authors did conclude that this high-affinity Mn-binding site was the native site for Mn functional in water oxidation on the basis of the observations that (1) the rate of DPC donation to PS II increased when functional Mn was removed (i.e., DPC cannot donate effectively in the presence of functional Mn), (2) the high-affinity site is observed only when functional Mn is removed from PS II membranes, (3) the affinity of Mn^{2+} for this site varied in the presence of different anions with a hierarchy similar to that observed for stimulation of O_2 evolution (Critchley et al., 1982), (4) the binding site was specific for Mn, (5) the affinity of Mn for the binding site decreased as the pH was increased from 6.1 to 7.7, and (6) the site was located on an intrinsic protein, more specifically on proteins of the isolated D1/D2 PS II reaction center complex (Seibert et al., 1989). Consistent with the above interpretation are results showing that half of the high-affinity Mn-binding site observed in spinach and *Scenedesmus obliquus* WT is not available in PS II membranes prepared from the O_2-evolution- and Mn-deficient LF-1 mutant of *Scenedesmus* (Seibert et al., 1989; Preston & Seibert, 1989). Furthermore, that part of the site missing in this mutant is identical with a DEPC-sensitive histidine site observed in WT *Scenedesmus* PS II membranes (Seibert et al., 1989), and this missing site can be restored through mild protease treatment of LF-1 PS II membranes (Preston & Seibert, 1989).

Tamura et al. (1989) suggested that there may be a relationship between EDC modification of PS II membranes and inhibition of Mn ligation. In this report we have investigated the possibility that the other half of the high-affinity Mn-binding site that is not sensitive to DEPC is composed of carboxyl residues. Carbodiimides have been used frequently to modify carboxyl groups of proteins [see Azzi et al. (1984) for a review of some applications], and here we employ the water-soluble carbodiimide EDC to modify half of the high-affinity binding site. The EDC-sensitive part of the high-affinity binding site was found to be distinct from and independent of the DEPC-sensitive part of the site. A preliminary report of this work is available (Preston & Seibert, 1990).

Experimental Procedures

Spinach PS II membrane fragments were prepared from market spinach by the method of Kuwabara and Murata (1982). *S. obliquus* WT and LF-1 cells were grown in 200-mL batches of NGY media (Bishop, 1971), and PS II enriched membranes were prepared as described by Metz and Seibert (1984). PS II membranes were resuspended in buffer A (20 mM MES-NaOH, pH 6.5, 0.4 M sucrose, 15 mM NaCl, and 5 mM $MgCl_2$) and stored at −80 °C until used. All PS II membrane preparations, with the exception of those used for $CaCl_2$ treatment, were depleted of Mn by treatment with Tris prior to use. PS II membranes were resuspended at 500 (μg of Chl)·mL^{-1} in 0.8 M Tris-HCl, pH 8.4, and 0.4 M sucrose (*Scenedesmus*) or 1.0 M Tris-HCl, pH 9.4, and 0.4 M sucrose (spinach) under room light at 4 °C for 30 min. The membranes were collected by centrifugation (10 min at 40000*g*), washed once in buffer A, and resuspended in the same buffer. The harsher Tris treatment was found to be necessary occasionally for complete depletion of Mn from spinach PS II membranes and therefore was used routinely.

PS II membranes at 0.5 (mg of Chl)·mL^{-1} were treated with 1 M $CaCl_2$, 20 mM MES-NaOH, pH 6.5, and 400 mM sucrose for 30 min in the dark to remove the extrinsic proteins but not Mn as described in Ono and Inoue (1983). The samples were collected by centrifugation (10 min at 30000*g*), washed once in buffer B (20 mM MES-NaOH, pH 6.5, 0.4 M sucrose, and 20 mM NaCl), and resuspended in the same buffer. The samples were aged in this buffer in the dark at 4 °C for 4 h. After aging, the samples were pelleted (10 min at 30000*g*), washed once in buffer B to remove released Mn, and resuspended in buffer A supplemented with an additional 175 mM NaCl (bringing the Cl^- concentration to 200 mM) to inhibit further Mn release (Ono & Inoue, 1985). EDC or DEPC treatment of the $CaCl_2$-washed PS II preparation was performed in the presence of 200 mM NaCl.

Modification of histidine residues with 500 μM DEPC was performed as described by Tamura et al. (1989). Although DEPC can potentially modify lysine, tyrosine, cysteine, serine, arginine, and α-amino groups of amino acids (Miles, 1977), Tamura et al. (1989) have provided chemical reversal, pK_a, inactivation rate constant, and control extrinsic protein data showing the DEPC specifically modifies histidine residues functional in photoligation of Mn and photoactivation under the conditions used in this paper. Modification of carboxyl groups with EDC was accomplished by incubating PS II membranes at 100 (μg of Chl)·mL^{-1} in buffer B containing 10 mM EDC for 1 h at 20 °C in the dark. The reaction was stopped by the addition of two volumes of buffer B containing 0.2 M CH_3COO·Na. The membranes were collected by centrifugation (10 min at 40000*g*), washed once in buffer B, and resuspended in buffer A. The specificity of the EDC reaction for carboxyl groups under these conditions will be examined later. When divalent cations were added to the EDC or DEPC incubation buffers, the PS II membranes were subjected to a second Tris treatment with 5 mM EDTA after the EDC or DEPC treatments to remove any extraneous cations that may have bound to the Mn-binding site.

Assays of DCIP photoreduction, used to monitor DPC/Mn^{2+} donation to PS II, were performed with an Aminco DW2a spectrophotometer in the split beam mode (Hsu et al., 1987; Seibert et al., 1989; Preston & Seibert, 1989). Actinic light from a slide projector was passed through heat (Melles

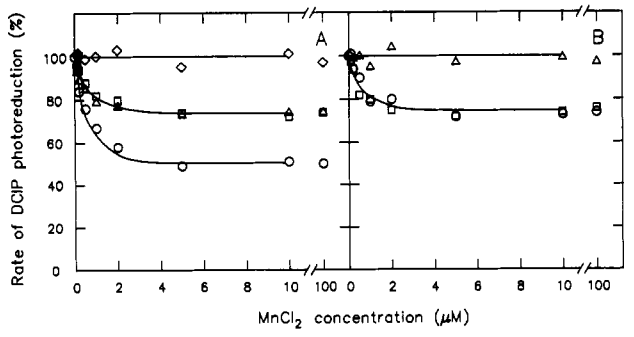

FIGURE 1: Influence of DEPC and EDC on the $MnCl_2$ inhibition of DPC-supported DCIP photoreduction of *S. obliquus* WT (A) and LF-1 (B) PS II membranes. Controls were Tris-treated PS II membranes (○). These membranes were then treated with 500 μM DEPC (□), 10 mM EDC (△), or 500 μM DEPC followed by 10 mM EDC (◇). 100% activities were 156, 140, 146, and 128 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated WT PS II membranes, the same membranes treated with DEPC, treated with EDC, and treated with DEPC then EDC, respectively; and 171, 150, and 159 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for control LF-1 PS II membranes, the same membranes treated with DEPC and treated with EDC, respectively.

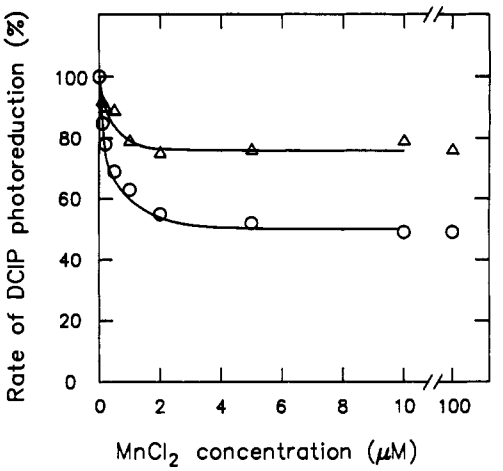

FIGURE 2: Influence of EDC on the $MnCl_2$ inhibition of DPC-supported DCIP photoreduction of spinach PS II membranes. Controls were Tris-treated PS II membranes (○), and these membranes further treated with 10 mM EDC (△). 100% activities were 275 and 258 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated PS II membranes and the same membranes treated with EDC, respectively.

Griot 03MHG007) and 630-nm cut-off (Schott RG630) filters and then focused onto the sample cuvette at 90° to the measuring beam. The light intensity at the sample cuvette was saturating (≥200 W·m^{-2}). The photomultiplier was protected from the excitation light with a 600-nm narrow band-pass filter (Melles Griot 03FIV045), and an extinction coefficient of 13 mM^{-1}·cm^{-1} (Armstrong, 1964) was used to calculate rates of DCIP photoreduction. DCIP photoreduction was monitored continuously, and rates were calculated by using the initial slope. The assay buffer was 50 mM MES-NaOH, pH 6.5, 0.4 M sucrose, 20 mM NaCl, 30 μM DCIP, and 200 μM DPC. Rates of DCIP photoreduction of control Tris-treated material ranged from 100–200 (μmol of DCIP)·(mg of Chl)$^{-1}$·h^{-1} for *Scenedesmus* WT and LF-1 PS II membranes and 200–400 (μmol of DCIP)·(mg of Chl)$^{-1}$·h^{-1} for spinach PS II membranes. $MnCl_2$ at micromolar concentrations was added to the assay buffer from 100 μM or 5 mM stocks prepared in assay buffer. At least two measurements were made for each $MnCl_2$ concentration in every experiment and averaged. The individual measurements were within 5% of each other. The data presented are representative of three to five replicate experiments. A further indication of the scatter of the experimental data can be obtained by comparing the same treatments in the different figures.

As mentioned above, the mechanism originally proposed to explain the above-mentioned assay (Hsu et al., 1987) is incorrect. We demonstrate in the Appendix that the correct mechanism involves noncompetitive inhibition and that potential side reactions between Mn^{3+} and either $DCIPH_2$ or DPC raised by Hoganson et al. (1989) have no significant influence on the assay. Furthermore, in the accompanying paper (Preston & Seibert, 1991), we present direct metal analysis evidence that more than one Mn can bind to the membrane under our assay conditions.

Results

The Mn-binding assay of Hsu et al. (1987) was used to determine the amount of high affinity Mn-binding site present in the various PS II membrane preparations. Figures 1 and 2 show typical $MnCl_2$ inhibition profiles for Mn-depleted *Scenedesmus* WT (Figure 1A), LF-1 (Figure 1B), and spinach (Figue 2) PS II membranes. Addition of micromolar concentrations of $MnCl_2$ to the DPC → DCIP assay buffer decreases activity for all three preparations. The maximum effect is seen with 5 μM $MnCl_2$, which decreases DPC → DCIP activity by 50% for WT and spinach membranes and by 25% for LF-1 membranes. Adding higher concentrations of $MnCl_2$, up to 100 μM, did not further increase the amount of inhibition. In previous reports (Seibert et al., 1989; Preston & Seibert, 1989), we showed that half of the high-affinity Mn-binding site could not be observed in LF-1 PS II membranes. Treatment of *Scenedesmus* PS II membranes with 500 μM DEPC decreases the $MnCl_2$ inhibition of DPC → DCIP activity of WT PS II membranes from 50% to 25% but does not affect DPC → DCIP activity of LF-1 membranes [see also Seibert et al. (1989) and Preston and Seibert (1989)]. Treatment of WT PS II membranes with the carboxyl modifier EDC also reduces $MnCl_2$ inhibition of DPC → DCIP activity from 50% to 25%. Unlike DEPC, EDC reduces $MnCl_2$ inhibition of DPC → DCIP activity of LF-1 PS II membranes from 25% to 0. The high-affinity Mn-binding site of spinach PS II membranes can also be modified with EDC (Figure 2), producing a result similar to that observed with WT PS II membranes. The two chemical modifiers each affect half of the high-affinity binding site in both WT and spinach membranes, and evidence from LF-1 membranes suggests that action of the two modifiers is mutually exclusive (i.e., they act on independent components of the site or independent sites). This conclusion is further strengthened when WT PS II membranes are modified first with DEPC and then with EDC (Figure 1A). After sequential modification, $MnCl_2$ was unable to inhibit DPC → DCIP activity at all, suggesting that Mn was unable to bind to the high-affinity site.

To determine whether EDC and DEPC were influencing the amount of high-affinity Mn-binding site through modification of the stromal or lumenal side of the membrane, we also modified spinach thylakoids with these compounds. The results are shown in Table I. In this table we have given the data as the fraction of DPC-supported photoreduction activity remaining in the presence of 10 μM $MnCl_2$ compared with activity in the absence of $MnCl_2$. From these results it can be seen that modification of the high-affinity Mn-binding site can occur only if the lumenal side of the membrane is exposed to the modifier.

In Figure 3 we have examined the concentration dependence for EDC modification of the high-affinity Mn-binding site. This figure plots the fraction of DCIP photoreduction activity remaining in the presence of 10 μM $MnCl_2$ against the EDC concentration used to modify the PS II membranes prior to

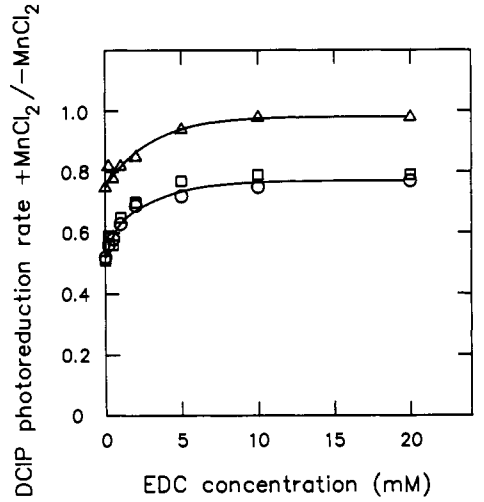

FIGURE 3: Concentration dependence of EDC modification of the high-affinity Mn-binding site. The ratio of DPC → DCIP activity in the presence of 10 μM $MnCl_2$ and in the absence of $MnCl_2$ in the assay buffer ($+MnCl_2/-MnCl_2$) is plotted against the concentration of EDC (1 h dark treatment at 20 °C) used to modify the PS II membranes prior to the assay. An increase in the ratio of $+MnCl_2/-MnCl_2$ corresponds to a decrease in the amount of high-affinity Mn-binding site available. Symbols: Tris-treated *Scenedesmus* WT (○), LF-1 (△), and spinach (□) PS II membranes.

Table I: Effects of Amino Acid Chemical Modification of Spinach Thylakoid Membranes on the High-Affinity Mn-Binding Site Compared to Those of Modified PS II Membrane Fragments[a]

sample and treatment	rate of DCIP photoreduction ($+MnCl_2/-MnCl_2$)
control PS II	0.52
control thylakoids/PS II	0.54
EDC-treated PS II	0.77
EDC-treated thylakoids/PS II	0.51
DEPC-treated PS II	0.74
DEPC-treated thylakoids/PS II	0.52

[a] EDC and DEPC modifications were performed as described under Experimental Procedures. After treatment, thylakoids (stromal side exposed, lumenal side sequestered) were fractionated into lumenal side exposed PS II membrane fragments (thylakoids/PS II), and the Mn was removed by Tris treatment for subsequent assay. PS II membrane fragments (PS II) were modified and Tris treated directly as described previously. DPC-supported DCIP photoreduction rates were measured in the presence and absence of 10 μM $MnCl_2$.

the assay. With *Scenedesmus* WT and spinach PS II membranes, the amount of activity increased from about 50% to about 77% with increasing EDC concentrations. With LF-1 PS II membranes, the amount of activity remaining increased from about 75% to about 98%. In all three cases, modification was complete at concentrations ≥10 mM EDC. There was no apparent difference in the concentration dependence of EDC modification in any of these preparations.

We have thus far established that EDC, as well as DEPC, modifies independent components of the high-affinity Mn-binding site. Figure 4A shows that the high-affinity Mn-binding site can be protected from EDC modification if $MnCl_2$ at millimolar concentrations is added to the EDC modification buffer. This is demonstrated when the material is subsequently assayed for DPC-supported DCIP photoreduction in the presence and absence of 10 μM $MnCl_2$. The results are plotted as the fraction of DCIP photoreduction activity remaining in the presence of 10 μM $MnCl_2$ against the concentration of $MnCl_2$ added to the EDC modification buffer. $MnCl_2$ was able to protect all three PS II membrane preparations against EDC modification of the high-affinity Mn-binding site with a similar $MnCl_2$ concentration dependence, and in all three cases 2 mM $MnCl_2$ was sufficient for complete protection. We also modified WT PS II membranes with 10 mM EDC in the presence of 5 mM $MnCl_2$ and then assayed for the amount of high-affinity Mn-binding site remaining (Figure 4B). The Mn^{2+} inhibition profiles were identical for the Tris-treated PS II membranes and membranes subsequently treated with EDC and $MnCl_2$. This supports our contention that $MnCl_2$ can provide complete protection against EDC modification. $MnCl_2$ was also successful in providing at least partial protection of the high-affinity Mn-binding site against DEPC modification (Figure 4A); however, a higher concentration of $MnCl_2$ (>10 mM) was required in this case. The difference between the EDC and DEPC experiments may be a consequence of the concentrations of modifiers used; however, other factors, which shall be discussed later (see next section), might be involved. The protection $MnCl_2$ affords against EDC modification of the high-affinity Mn-binding site is specific for Mn^{2+} as none

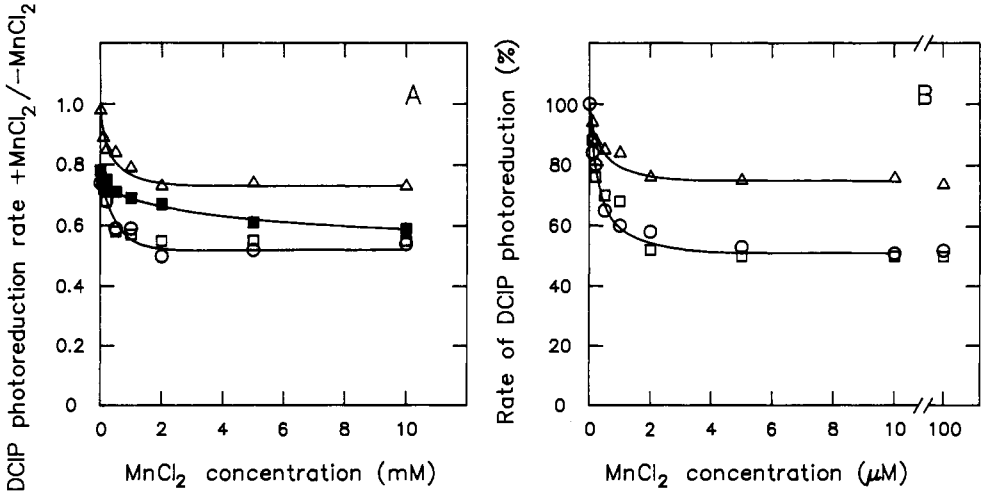

FIGURE 4: $MnCl_2$ protection of the high-affinity Mn-binding site against EDC and DEPC modification in Tris-treated PS II membranes. (A) Ratio of DPC → DCIP activity in the presence of 10 μM $MnCl_2$ and in the absence of $MnCl_2$ in the assay buffer ($+MnCl_2/MnCl_2$) plotted against the concentration of $MnCl_2$ added to the EDC modification buffer. 10 mM EDC was used to modify all samples: *Scenedesmus* WT (○), LF-1 (△), and spinach (□) PS II membranes. The same experiment with spinach PS II membranes is also shown except that $MnCl_2$ was used to protect against DEPC (500 μM) modification of the high-affinity Mn-binding site (■). (B) $MnCl_2$ inhibition of DPC-supported DCIP photoreduction of WT PS II membranes. Controls were Tris-treated PS II membranes (○). The same membranes were then treated with 10 mM EDC (△) or 10 mM EDC in the presence of 5 mM $MnCl_2$ (□).

Table II: EDC Modification of the High-Affinity Mn-Binding Site of Spinach PS II Membranes in the Presence of Divalent Cations

cation added	rate of DCIP photoreduction ($+MnCl_2/-MnCl_2$)	cation added	rate of DCIP photoreduction ($+MnCl_2/-MnCl_2$)
no addition	0.74	5 mM $ZnCl_2$	0.72
5 mM $MnCl_2$	0.52	5 mM $CuCl_2$	0.79
5 mM $CaCl_2$	0.75	5 mM $MgCl_2$	0.76
5 mM $BaCl_2$	0.73	5 mM $CoCl_2$	0.79

[a] Tris-treated PS II membranes from spinach were incubated in buffer B with 10 mM EDC for 1 h. To this incubation medium, additional salts were added as indicated. The DPC → DCIP assay was performed in the presence and absence of 10 μM $MnCl_2$, and the ratios of the two results are given.

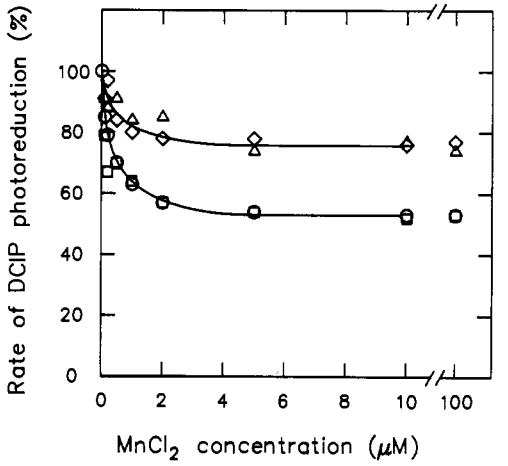

FIGURE 5: Influence of DTT on EDC modification of the high-affinity Mn-binding site in spinach PS II membranes. $MnCl_2$ inhibition of DPC-supported DCIP photoreduction is shown in Tris-treated PS II control membranes (○). The same membranes were treated with 0.5 mM DTT (□), 10 mM EDC (△), or 10 mM EDC in the presence of 0.5 mM DTT (◇). PS II membranes were incubated for 5 min in the presence or absence of DTT. The incubation was then continued for an additional 1 h in the presence or absence of EDC. 100% activities were 320, 310, 283, and 298 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated PS II membranes and for these membranes treated with DTT, EDC, or both DTT and EDC, respectively.

of the other divalent cations tested was able to protect against EDC modification (Table II).

As well as modifying carboxyl residues, carbodiimides can also modify cysteine residues at neutral pH (Azzi et al., 1984). According to the folding model of the D1 and D2 proteins proposed by Trebst (1986), there is only one cysteine residue (Cys 72 on D2) on the lumenal side of the PS II membrane. We investigated whether modification of this residue by EDC affects the high-affinity Mn-binding site by performing the EDC modification in the presence and absence of 0.5 mM DTT (Figure 5). Addition of the DTT should lead to protection of the thiol groups from attack by EDC (Azzi et al., 1984). We were unable to detect a difference in the amount of high-affinity Mn-binding site modified by EDC under the two conditions. Addition of DTT to the control incubation likewise did not affect the amount of Mn-binding site.

EDC is a successful cross-linker of carboxyl and amino groups (Azzi et al., 1984). EDC first reacts with a carboxyl group forming in *O*-acylisourea derivative. This derivative is unstable and is likely to react with a nearby amino group resulting in cross-linking the two groups. Thus it is important to determine whether it is the carboxyl or the amide group of the potential cross-link that is the high-affinity Mn-binding site being modified by EDC. We attempted to alter the amount of potential cross-linking occurring during incubation with EDC in two ways. First, we reduced the pH of the incubation buffer to 5.0 during the EDC modification since

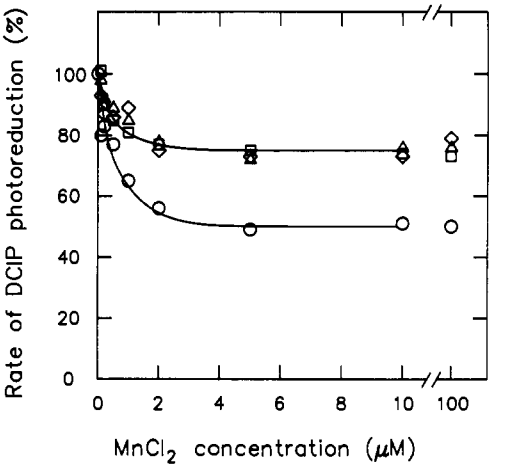

FIGURE 6: Influence of pH and CH_3NH_2 on EDC modification of the high-affinity Mn-binding site in spinach PS II membranes. $MnCl_2$ inhibition of DPC-supported DCIP photoreduction is shown in Tris-treated PS II control membranes (○). The same membranes were treated with 10 mM EDC at pH 6.5 (△), 10 mM EDC at pH 5.0 (□), and 10 mM EDC in the presence of 50 mM CH_3NH_2 at pH 5.0 (◇). During the modification procedure, buffer B was adjusted to pH 5.0 with HCl. The pH of this incubation was monitored periodically and readjusted to pH 5.0 with HCl as required. 100% activities were 275, 234, 152, and 161 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated PS II membranes and for the same membranes treated with EDC at pH 6.5, with EDC at pH 5.0, and with EDC at pH 5.0 in the presence of CH_3NH_2, respectively.

the cross-linking reaction between carboxyl and amino groups is most effective at this pH (Hoare & Koshland, 1967). The second technique used was to incubate PS II membranes with EDC in the presence of 50 mM CH_3NH_2 at pH 5.0. Added amines have been successfully used to modify carboxyl groups of proteins by this cross-linking reaction (Kramer & Rupley, 1973; Yamada et al., 1981). The second experiment should promote modification of carboxyl residues with CH_3NH_2 and inhibit cross-linking to other amino acids. In neither case did we see any change in the $MnCl_2$ inhibition of DPC → DCIP activity (Figure 6). This suggests that carboxyl and not amino groups are involved in the EDC modification of the high-affinity Mn-binding site (see Discussion).

$CaCl_2$ treatment of PS II membranes removes the three extrinsic proteins of 33-, 23-, and 18-kDa from the membrane but does not affect functional Mn (Ono & Inoue, 1983). If the $CaCl_2$-extracted membranes are allowed to age in a buffer containing low concentrations of NaCl (<50 mM) and no Ca^{2+}, loss of functional Mn occurs (Ono & Inoue, 1985) in parallel with loss of O_2 evolution activity (Seibert et al., 1988). About half of the Mn will be lost during a 4–6 h incubation (Ono & Inoue, 1985). Figure 7 shows the results of an experiment where the amount of Mn bound to the membrane was manipulated through aging of $CaCl_2$-treated PS II membranes. In this instance the PS II membranes were not Tris treated. $MnCl_2$ was unable to inhibit DPC → DCIP activity when PS II membranes, treated with $CaCl_2$, were assayed immediately, indicating that the full complement of functional Mn was still present. After the $CaCl_2$-treated membranes were aged for 4 h and washed to remove released Mn, added $MnCl_2$ was able to inhibit DPC → DCIP activity by 25%. Thus, aging released about half of the functional Mn from the membrane, which resulted in the appearance of about half of the high-affinity Mn-binding site. The aged $CaCl_2$-treated material was subsequently incubated with EDC to identify the site that appeared as a result of aging. Since $MnCl_2$ was unable to inhibit DPC → DCIP activity of this material, all of the high-affinity Mn-binding site was protected (half by functional

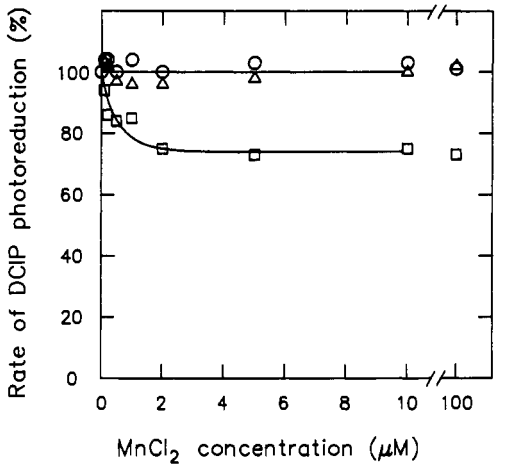

FIGURE 7: Mn lost through aging of $CaCl_2$-treated spinach PS II membranes comes from the EDC-sensitive high-affinity Mn-binding site. $MnCl_2$ inhibition of DPC-supported DCIP photoreduction of PS II membranes treated with 1 M $CaCl_2$ and assayed immediately prior to aging (○). The same membranes after aging in low-salt buffer (see Experimental Procedures) for 4 h (□). Aged $CaCl_2$-treated PS II membranes after further treatment with 10 mM EDC (△). 100% activities were 122, 176, and 156 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for control $CaCl_2$-treated PS II membranes, for the same membranes aged 4 h, and for those aged 4 h with subsequent EDC treatment, respectively.

Mn still bound to the membrane and the other half by EDC modification). Aged $CaCl_2$-treated PS II membranes were also treated with DEPC. In this experiment we were unable to detect any effect of DEPC (data not shown), which indicates that the available high-affinity sites in aged $CaCl_2$-treated material are not associated with histidines. Thus, the loosely bound component of the functional Mn pool in PS II covers or is bound to the EDC-sensitive high-affinity Mn-binding site.

Discussion

Tamura et al. (1989) used the histidine-modifying reagent DEPC to show that histidine residues on D1 are required for Mn photoligation and photoactivation of Mn-depleted PS II membranes. In other studies (Seibert et al., 1989; Preston & Seibert, 1989), DEPC was also found to inhibit the high-affinity Mn-binding site in PS II. Furthermore, the latter studies demonstrated that the high-affinity Mn-binding site was composed of two equal parts, one sensitive to and one insensitive to DEPC. Recent thermoluminescence studies suggest that histidine(s) provides a redox-active ligand for Mn in Tris-treated PS II membranes (Ono & Inoue, 1991). We now report that the DEPC-insensitive half of the high-affinity Mn-binding site is sensitive to EDC, a carboxyl modifier (Figure 1). We also show that EDC modification of the high-affinity Mn-binding site can be inhibited specifically by Mn^{2+} (Figure 4, Table II), indicating that EDC is indeed modifying amino acids that are able to bind Mn and not other cations.

The reaction of carbodiimides with carboxyl groups initially produces an unstable *O*-acylisourea derivative. This derivative can then react through three possible pathways (Hoare & Koshland, 1967; Carraway & Koshland, 1972). The first pathway is a reaction with H_2O that releases the carbodiimide as a urea and regenerates the carboxyl group with no net modification. The second is a reaction with a nearby amino group that also releases the carbodiimide as its urea but in this case leads to cross-linking of the carboxyl and amino groups. The third reaction is a rearrangement of the *O*-acylisourea derivative leading to stable binding of the carbodiimide. Since we observe a permanent modification of carboxyl groups by EDC, the first reaction is of little importance in our case. Changing the EDC incubation conditions by lowering the pH increases the probability of the second reaction. However, no change in the amount of high-affinity Mn-binding site affected is observed under these conditions (Figure 6). We also added CH_3NH_2 to the incubation buffer, which should complete with the second reaction. Again we found no effect on EDC modification of the high-affinity Mn-binding site. Moreover, CH_3NH_2 should also compete with H_2O for the *O*-acylisourea derivative in the first reaction, leading to a greater number of carboxyls being modified. We can therefore conclude that (1) all of the carboxyls that are available for EDC modification, that are part of the high-affinity Mn-binding site, and that are detectable by the DPC/Mn^{2+} assay are probably being modified; and (2) any amino groups cross-linked by EDC do not contribute to the Mn-binding site. Additionally, the DTT experiments (Figure 5) indicate that potential EDC modification of cysteine residues does not affect the high-affinity Mn-binding site.

A greater concentration of $MnCl_2$ (>10 mM) was required to protect the high-affinity Mn-binding site from modification by DEPC compared to EDC (Figure 4). Two factors may contribute to this effect. The first is related to the concentration of DEPC. Successful modification of histidine residues has been achieved in other proteins with DEPC concentrations as low as 75 μM (Leskovac & Pavkov-Pericin, 1975) whereas 500 μM was used in these experiments. The second is a consequence of the reaction mechanisms for the two chemical modifiers. The DEPC reaction is essentially irreversible unless NH_2OH is present (Miles, 1977), whereas EDC modification can be reversed with H_2O. This provides a greater opportunity for Mn^{2+} to associate with the Mn-binding site during EDC modification than during DEPC modification. Tamura et al. (1989) found that 1 mM $MnCl_2$ added to the DEPC modification buffer reduced the inhibition of photoactivation by DEPC by a significant amount if the incubation was performed in the light, but not in the dark. This can easily be rationalized with our data since Mn is bound more tightly in the light than in the dark (Tamura & Cheniae, 1987). Therefore, Mn^{2+} can more easily compete with DEPC under photoactivating conditions than in our experiments where DEPC modification occurs in the dark.

Considerable evidence suggests that half of the functional Mn of PS II is more labile upon the removal of the 33-kDa protein than the other half (Ono & Inoue, 1985; Seibert & Cotton, 1985; Cole et al., 1987; Seibert et al., 1988). Here we are able to equate the more loosely bound pool of functional Mn with the EDC-sensitive Mn-binding site (Figure 7). In these experiments we observe that, after being aged under conditions that should lead to loss of 50% of the functional Mn (Ono & Inoue, 1985; Cole et al., 1987; Seibert et al., 1988), half of the high-affinity Mn-binding site becomes available. This observation provides additional evidence that the high-affinity Mn-binding site we observe is closely related to the functional Mn-binding site. There also appears to be a heterogeneity associated with the functional Mn in that the Mn bound to histidine residues is more stable than that bound to carboxyl residues. It is tempting to speculate that it is the ligand environment of the Mn bound to histidine residues that is making these Mn more stable. In another example of Mn heterogeneity, Klimov et al. (1982) proposed that half of the functional Mn in PS II could be replaced by Mg^{2+} or other divalent cations under some conditions. Perhaps it is the more loosely bound Mn that can be replaced by other cations, but as yet we have no evidence on this point.

This study has determined that the two amino acid modifiers (DEPC and EDC) do not overlap in action (as far as detectable by the DPC/Mn^{2+} assay) and therefore must affect different components of the high-affinity Mn-binding site located on the lumenal side of the membrane (Table I). Evidence from EXAFS studies suggests that there are two to four terminal ligands (i.e., ligands from protein to Mn) to each Mn that are either N or O (Kirby et al., 1981; Yachandra et al., 1986; George et al., 1989). No evidence was found for S ligands although a small contribution from these ligands could not be ruled out. One interpretation of our data suggests that histidine residues provide terminal ligands exclusively to half of the high-affinity Mn, and carboxyl groups provide ligands to the other half. There are a large number of carboxyl residues on lumenal-side peptide constituents of the isolated PS II D1/D2 reaction center complex. However, depending on whether a model of 1, 2, or more Mn constitute the high-affinity Mn-binding site, and whether histidines bind other prosthetic groups, there may be insufficient histidine residues on the lumenal exposed portions of D1 and D2 for all of the terminal ligands to Mn (note that the other three smaller PS II reaction center peptides do not contain lumenal-side histidines). This suggests that there may be other ligands to Mn that we are unable to observe and leads to a second explanation of our data. The assay for the high-affinity Mn-binding site may identify only one of two to four possible terminal ligands to each Mn. At present we are unable to distinguish between these two possibilities, but evidence from experiments with proteases and metal analyses in the accompanying paper (Preston & Seibert, 1991) leads us to consider the second explanation.

The EXAFS data cited above are unable to distinguish between N and O as terminal ligands to Mn. However, Andréasson (1989) has suggested that there are no N ligands to Mn in the S_2 state of the oxygen-evolving system. This assertion is based on EPR multiline studies of PS II membranes isolated from ^{15}N-grown spinach, which revealed little change in the fine structure of the EPR signal on replacement of ^{14}N by ^{15}N. The above conclusion is at variance with studies using the histidine chemical modifier DEPC (Tamura et al., 1989; Seibert et al., 1989; Preston & Seibert, 1989; Ono & Inoue, 1991; the present study), which suggest that there are N ligands to Mn. Several explanations for this discrepancy are possible. First, the DEPC-sensitive high-affinity Mn-binding site may not be part of the functional Mn site required for O_2 evolution. If this is the case, at least some of the experiments in this study may be probing a Mn-binding site (i.e., a Mn photoligation site) prior to "the site" formed after complete incorporation of Mn into the oxygen-evolving complex. This would imply a multi-step binding process in which ligands to Mn change during the photoactivation process. A multistep process has been described (Tamura & Cheniae, 1987; Miller & Brudvig, 1990) during photoactivation; however, no evidence for movement of Mn atoms from the initial binding site to another site has been reported. Second, it is not yet clear how the Mn atoms interact to produce the EPR multiline signal (Hoff, 1987), and also the form and number of lines are dependent on experimental conditions such as illumination temperature and time of dark adaptation (de Paula et al., 1985). As a result, the multiline EPR signal may reflect only the Mn that is not bound to histidine residues. Third, Hansson et al. (1986) found that replacing $H_2{}^{16}O$ with $H_2{}^{17}O$ only results in a small broadening of the fine structure of the multiline signal. If the signal due to this isotopic replacement, in a molecule that is presumably intercalating between the Mn atoms, is not large, then isotopic replacement at a peripheral ligand might also have only a small effect. Further to this point, Britt et al. (1989) have shown that binding of $^{15}NH_4Cl$ to the Mn of the oxygen-evolving system produced the same multiline EPR signal as the binding of $^{14}NH_4Cl$; whereas using a more sensitive EPR technique, electron spin-echo envelope modulation (ESEEM) spectroscopy, they were able to clearly demonstrate a difference in the signal when $^{15}NH_4Cl$ or $^{14}NH_4Cl$ was bound to the Mn of PS II. This indicates that N isotopic effects on the EPR multiline signal are difficult to observe. De Rosa et al. (1991) recently confirmed this conclusion and, by comparing ESEEM spectra of O_2-evolving PS II complexes from *Synechococcus* grown on $^{14}NO_3^-$ or $^{15}NO_3^-$, went on to show that there were N ligands to Mn in PS II. From this and previous arguments, we conclude that the most likely explanation for our data is that DEPC and EDC modify histidine and carboxyl residues that are ultimately involved in binding Mn functional in O_2 evolution.

At this point it is useful to consider the mechanism involved in the high-affinity Mn-binding assay, since the work of Hsu et al. (1987) has been questioned by Hoganson et al. (1989). First of all, we show in the Appendix that at pH 6.5 the assay mechanism involves noncompetitive inhibition by Mn of light-driven DPC → DCIP electron transport rather than competitive inhibition (Hsu et al., 1987). We also show that the assay is not influenced significantly by side reactions between Mn^{3+} and either $DCIPH_2$ or DPC (Appendix). We conclude that under our conditions (high DPC concentration range, Figure 8) Mn, at least in the steady state, is not acting *predominantly* as a donor to PS II (since we show in the Appendix that only about one Mn^{3+} per reaction center is photoproduced on an average every second under continuous illumination), but, by binding to the membrane, it is actually preventing DPC donation. The bound form of Mn that actually inhibits DPC donation may be minimal Mn^{3+} formed rapidly when illumination commences. Binding of DPC and Mn in this case is at different sites. The fact that DPC donation is decreased by only 50% rather than 100% under saturating light conditions with as little as 5 μM Mn^{2+} present initially, suggests that DPC can donate to PS II through two intrinsic donors and that Mn binding prevents donation to only one of them.[2] This is consistent with the work of Blubaugh and Cheniae (1990) and Tamura et al. (1990), who show that DPC can donate to PS II through both Z and an alternative site that is probably D. Photoinhibition studies indicate that Mn under the conditions of the assay inhibits DPC donation to Z but not to D (D. J. Blubaugh and G. M. Cheniae, personal communication). Superficially, functioning of Mn *predominantly* as an inhibitor appears to conflict with the single-turnover flash studies of Hoganson et al. (1989), who show that one Mn^{2+} can donate directly to the secondary donor Z. However, Mn^{2+} does donate to PS II under the conditions of the DPC/Mn^{2+} assay if the DPC concentration is in the low concentration range of Figure 8 in the Appendix (see also Table III in the Appendix). In this situation, both DPC and Mn^{2+} probably donate through Z only, on the basis of K_M

[2] The mechanism cannot involve the complete inhibition of DPC donation to PS II by 5 μM Mn^{2+} and concurrent donation by Mn^{2+} to PSII. In Table III, we show that the rate of DCIP photoreduction with Mn-depleted spinach PS II membranes is about 40 μmol·(mg of Chl)$^{-1}$·h^{-1} in the presence of 5 μM Mn^{2+} (and no DPC), whereas the rate is 275 μmol·(mg of Chl)$^{-1}$·h^{-1} in the presence of 200 μM DPC (and no Mn^{2+}). If the above were the operative mechanism, one would expect DCIP photoreduction to decrease to about 15% of the control level when Mn^{2+} is included along with DPC, which is not the case.

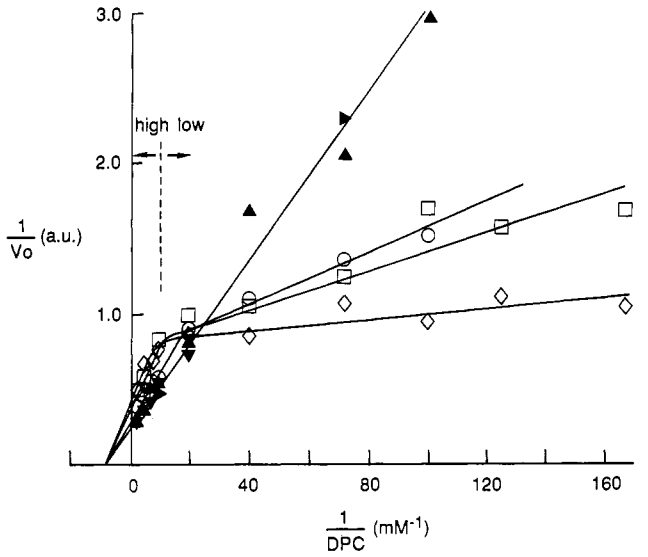

FIGURE 8: Lineweaver–Burk plot of Mn^{2+}-induced inhibition of DPC-supported DCIP photoreduction in NH_2OH-treated spinach PSII membrane fragments. The vertical dashed line divides what we term the high DPC and low DPC concentration ranges. The mechanism of interaction between DPC and Mn^{2+} is different in the two DPC concentration ranges. At high concentration (the conditions of the DPC/Mn^{2+} high-affinity assay and those used in this study), Mn^{2+} is a noncompetitive inhibitor of DPC whereas at low concentrations DPC and Mn^{2+} appear to donate to PS II additively. $MnCl_2$ concentrations: 0 μM (solid triangles), 0.3 μM (○), 1 μM (□), 5 μM (◇). The chlorophyll concentration was 5 μg/mL.

arguments (Blubaugh & Cheniae, 1990) and EPR studies (Hoganson et al., 1989; Miller & Brudvig, 1990). Thus, the interaction of Mn^{2+} with PS II is complex and dependent on the concentration of DPC present. Whether this is the case with donors other than DPC is yet to be determined. Consequently, direct comparison of the work of Hoganson et al. (1989) with that of Hsu et al. (1987) is difficult, and the points raised by Hoganson et al. (1989), while valid under their conditions, are not applicable when high concentrations of DPC are present in addition to Mn^{2+}.

We have interpreted our data in terms of binding of Mn to histidine or carboxyl residues that can be modified with DEPC or EDC. Other interpretations are of course possible, such as modifier effects at sites remote from the actual site of Mn binding. Such an effect might alter protein conformational changes induced by photoligation of Mn. In such a scenario, modification of remote histidine and carboxyl residues would have to inhibit conformational changes leading to an apparent reduction in the amount of Mn-binding sites. As yet we have no way of observing such conformational changes. However, to account for our data, we must postulate at least two separate remote conformational change effects, one blocked by EDC and a second blocked by DEPC.[3] Furthermore, we show in Table II that only Mn^{2+} can *protect* the high-affinity Mn-binding site from EDC modification, which indicates that the EDC modification site has a specific association with Mn^{2+} (i.e., Mn^{2+} must bind to the membrane at the specific amino acid site that can be modified). Therefore, the simplest interpretation of our data is that EDC [and DEPC, see Tamura et al. (1989) and Figure 4] directly modifies a Mn-binding site. Also, the known ligation pattern of Mn in other proteins, which shows a mixed carboxyl/histidine pattern [Hughes and Williams (1988) and see also Miller and Brudvig (1990)] is consistent with our interpretation. We therefore conclude that EDC and DEPC modifications are affecting residues to which Mn binds directly. This in turn leads to the proposal that there is a heterogeneity of Mn-binding sites in PS II, half of which are associated with histidine residues and the other half associated with carboxyl residues. In the accompanying paper (Preston & Seibert, 1991), we will present evidence suggesting that more than one

[3] In actuality, four separate conformational changes must be postulated since, as shown in the accompanying paper (Preston & Seibert, 1991), both the EDC- and DEPC-sensitive sites consist of two components each.

Table III: Initial Rates of DCIP Photoreduction with NH_2OH-Treated Spinach PS II Membranes (5 μg of Chl/mL) in the Presence of Different Donors[a]

donor	rate of DCIP photoreduction [(μmol of DCIP reduced)·(mg of Chl)$^{-1}$·h^{-1}]
(1) 5 μM Mn^{2+}	42
(2) 10 μM DPC	97
(3) both 5 μM Mn^{2+} and 10 μM DPC	133
(4) 1 plus 2	139
(5) 200 μM DPC	275

[a] Note that the concentration of DPC is in the low concentration range seen in Figure 8. In this concentration range, Mn and DPC donation is additive, demonstrating a mode of DPC/Mn^{2+} interaction that is different from that observed at high DPC concentration, the condition used in assaying for high-affinity Mn in this paper.

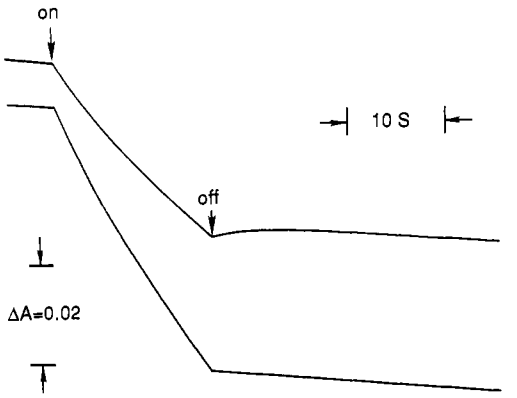

FIGURE 9: Kinetics of DPC-supported DCIP photoreduction and dark reoxidation in the presence (top) and absence (bottom) of 10 μM $MnCl_2$. The DPC concentration was 200 μM. The sample material in this and subsequent figures was the same as in Figure 8.

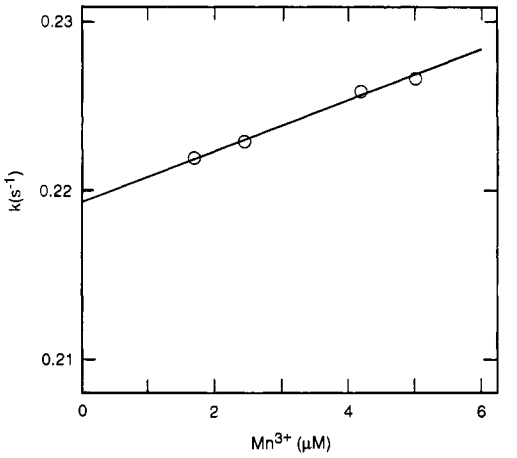

FIGURE 10: Plot of the pseudo-first-order rate constants for dark $DCIPH_2$ reoxidation obtained in the presence of 200 μM DPC after different illumination times (which builds up different amounts of Mn^{3+}) as a function of the expected amount of Mn^{3+}. Mn^{3+} was estimated from the amount of DCIP photoreduced under the same conditions (10 μM Mn^{2+} present initially) except that DPC was omitted from the assay medium. The rate constant k_1 for reaction 1 was obtained from the slope of the curve.

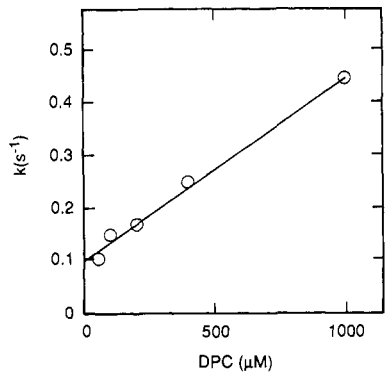

FIGURE 11: Plot of the pseudo-first-order rate constants for dark $DCIPH_2$ reoxidation as a function of the initial DPC concentration after an illumination period of 15 s. The concentration of Mn^{2+} present at the beginning of the illumination period was 10 μM. Under these conditions the maximum steady-state level of Mn^{3+} that could be present at the end of the illumination period was about 3 μM. This value was obtained by determining the amount of Mn^{3+} formed in the absence of DPC when 10 μM $MnCl_2$ was present at the time the light was turned on. Since 3 μM Mn^{3+} is much lower than any of the DPC concentrations used in this plot, one can obtain k_2 for reaction 2 from the slope of the curve.

Mn per reaction center can bind to the membrane under our assay conditions and outline some possible locations for the high-affinity Mn-binding site identified in this study. Alternative explanations will also be discussed.

ACKNOWLEDGMENTS

We thank Dr. Shinichi Taoka for helping us clarify the mechanism of the DPC/Mn^{2+} assay and Mr. Stephen Toon for preparing some of the PS II membrane fragments. We also appreciate the helpful suggestions of Dr. Irwin Fridovich, and the willingness of Drs. D. J. Blubaugh and G. M. Cheniae to share their unpublished results regarding Mn inhibition of DPC to Z electron transport with us.

APPENDIX

Hsu et al. (1987), relying on Lineweaver–Burk plots, described the mechanism of their assay as a competitive inhibition by Mn^{2+} of DPC-supported DCIP photoreduction. We question this interpretation because we found that either DEPC- or EDC-treatment eliminates half of the high-affinity Mn-binding site but that neither had a dramatic effect on the initial rate of DPC-supported DCIP photoreduction in control versus modified material in the absence of $MnCl_2$ (see the figures in the main text). This suggests a mechanism requiring different binding sites for DPC and Mn^{2+} interaction rather than the same site for both as Hsu et al. (1987) concluded.

To investigate this discrepancy, we repeated the Hsu et al. (1987) Lineweaver–Burk study under a much broader range of DPC and $MnCl_2$ concentrations. The sample material was 5 mM NH_2OH-treated spinach PS II membranes washed three times by centrifugation (Seibert et al. 1989), and the light intensity was saturating. Figure 8 shows that in the DPC concentration range used for our assays (high concentration), the mechanism is in fact noncompetitive inhibition, consistent with our other data (see above). We have been able to simulate the data of Hsu et al. (1987) in this DPC concentration range by using old DCIP stock solution in the assay (data not shown). Under this condition, we found that unique determination of the initial rates required for obtaining the double-reciprocal plots was difficult. This difficulty could have led to the previous erroneous mechanism. Figure 8 also implies a different mode of DPC and $MnCl_2$ interaction in the low DPC concentration range. Here, DPC and Mn^{2+} donation seems to be additive (see Table III).

While it appears that, at high DPC concentration, the presence of micromolar concentrations of Mn^{2+} prevents half of the normal rate of DPC donation to PS II, Mn^{2+} can also be a donor to PS II under some conditions as seen in Table III. Therefore, we considered the possibility that the DPC/Mn^{2+} assay (even at high DPC concentration) might be complicated by side reactions resulting from the multiple turnovers that may be required for measuring initial rates of DCIP photoreduction. A comparison of the kinetics of DCIP phototreduction and dark reoxidation with 200 μM DPC in the presence and absence of an initial concentration of 10 μM Mn^{2+} (Figure 9) shows in fact that some Mn^{3+} may build up after continuous illumination (i.e., in the former case there is dark reoxidation of reduced DCIP). This means that one or both of the following reactions may occur to some extent in the dark:

$$2Mn^{3+} + DCIPH_2 \xrightarrow{k_1} 2Mn^{2+} + DCIP + 2H^+ \quad (1)$$

$$Mn^{3+} + DPC \xrightarrow{k_2} Mn^{2+} + DCP_{ox} \quad (2)$$

If k_1 and k_2 are large compared to the rate constants for DPC and Mn^{2+} photooxidation ($>10^6$ $M^{-1}\cdot s^{-1}$; Hoganson et al., 1989), the apparent initial rate of DCIP reduction may be affected by back-reactions, thus potentially interfering with the assay.

To exclude this possibility, we obtained k_1 by plotting pseudo-first-order rate constants for $DCIPH_2$ reoxidation in the presence of both DPC and Mn^{2+} after different illumination times, assuming that the amount of Mn^{3+} present at the end of illumination was the same as that built up when 10 μM Mn^{2+} (but no DPC; however, see below) was present in the assay when the light was first turned on. The slope of the pseudo-first-order rate constants versus Mn^{3+} concentration (Figure 10) gave an a value of 1.5×10^3 $M^{-1}\cdot s^{-1}$ for k_1. The assumption above about the amount of Mn^{3+} present at the end of the illumination period (ca. 50–200 Mn^{3+}/reaction center) may be an overestimate. Miller and Brudvig (1990), for example, report the generation of three bound Mn^{3+} ions per reaction center under continuous light in Mn-depleted PS II membrane fragments subjected to photoactivating conditions. Since we are not exposing our samples to photoactivating conditions, which eliminates Mn^{2+} donation to PS II as O_2-evolution capacity appears, the real concentration of Mn^{3+} that results in our experiments may be somewhere in between the above limits. Because an overestimate of the Mn^{3+} concentration would decrease the apparent value of k_1, we also measured k_1 directly in the presence of 5–10 μM Mn^{2+} (but in the absence of DPC) after continuous illumination for 15 s. The k_1 value obtained in this manner was $(1.2–1.5) \times 10^4$ $M^{-1}\cdot s^{-1}$. If this is the true value of k_1, the k_1 obtained from Figure 10 above implies that, in the presence of 200 μM DPC, 12 Mn^{3+} per reaction center are generated by Mn-depleted PS II membranes exposed to saturating light for 15 s. It is also possible that this value of k_1 might be an underestimate if the rate constant for Mn^{3+} reduction by O_2^- or H_2O_2 (Archibald & Fridovich, 1982) is of the order of 10^4 $M^{-1}\cdot s^{-1}$ or higher. Since PS II is known to produce both O_2^- and H_2O_2 under some conditions, we tested the possibility by examining the effect of superoxide dismutase and catalase on the results of a DPC/Mn^{2+} assay. Neither enzyme increased the initial rate of DCIP photoreduction or dark reoxidation when both DPC and Mn^{2+} were present in the assay. Furthermore, there was no effect on the ratio of the initial rates of DCIP photoreduction in the presence and absence of 10 μM Mn^{2+} (i.e.,

the expected ratio of about 0.5 did not change when each enzyme was added; see the control value in Table I). Mn^{3+} can also disproportionate to Mn^{2+} and MnO_2 in aqueous solution, which could also cause us to overestimate the amount of Mn^{3+} present. However, MnO_2 is an insoluble black precipitate at around neutral pH. No precipitate was observed to form during a DPC/Mn^{2+} assay even when the Mn^{2+} concentration was increased to between 100 μM and 1 mM. Consequently, we believe that Mn^{3+} disproportionation is not affecting the assay and k_1 is around 1.5×10^4 $M^{-1}\cdot s^{-1}$.

We obtained k_2 by plotting pseudo-first-order constants for $DCIPH_2$ reoxidation in the dark as a function of the initial DPC concentration (Figure 11). The slope of the curve gives a value of 3.2×10^2 $M^{-1}\cdot s^{-1}$ for k_2 with the assumption made in the legend to Figure 11. Using the correction factor obtained above (i.e., ca. 10), we obtain 3.2×10^3 $M^{-1}\cdot s^{-1}$ for k_2. We conclude that both k_1 and k_2 are small compared to the rate constants for the photoreactions discussed above; thus, reactions 1 and 2 should not significantly affect the DPC/Mn^{2+} assay.

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