Protease Treatments of Photosystem II Membrane Fragments Reveal That There Are Four Separate High-Affinity Mn-Binding Sites†

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ABSTRACT: The "high-affinity Mn-binding site" in Mn-depleted photosystem II (PS II) membrane fragments isolated from *Scenedesmus obliquus* was examined by using the diphenylcarbazide (DPC)/Mn^{2+} noncompetitive inhibition assay [Preston, C., & Seibert, M. (1991) *Biochemistry* (preceding paper in this issue)]. Different proteases were used to degrade lumenal surface protein segments from these PS II membranes, and a total of four independent high-affinity Mn-binding sites (ligands) were identified. Carboxypeptidase A, subtilisin, and *Staphylococcus aureus* V8 protease each degrade one of two high-affinity Mn-binding sites sensitive to the histidine chemical modifier diethyl pyrocarbonate (DEPC). However, sequential treatment experiments indicate that subtilisin degrades a DEPC-sensitive Mn-binding site that is different from the one degraded by the other two proteases. Trypsin also was found to degrade one of the DEPC-sensitive Mn-binding sites (that degraded by carboxypeptidase A and V8 protease). In addition, trypsin degrades one of two 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC) sensitive Mn-binding sites, but only in the absence of the 30-kDa extrinsic protein. Thus, the 30-kDa extrinsic protein associated with O_2 evolution appears to protect the EDC-sensitive binding site from trypsin degradation. No protease has yet been identified that will degrade the trypsin-insensitive EDC-sensitive Mn-binding site. Under the conditions of the assay (high DPC concentration), more than three Mn per reaction center were found bound to the membrane with a K_M of about 0.4 μM, as determined by direct metal analysis. This is consistent with the idea that each of the four high-affinity sites binds (or provides a ligand for) one of four Mn. These four high-affinity Mn-binding sites may correspond to the four sites formed, albeit sequentially, in the normal photoactivation process. We provide evidence that a histidine residue (His 337) on D1 is a prime candidate for one of the high-affinity Mn-binding sites and suggest that it also provides a nitrogen ligand for binding functional Mn. Finally, an alternative explanation of our data might be that the four high-affinity sites represent four ligands binding a single Mn, and ligands to other membrane bound Mn are not detectable by the DPC/Mn^{2+} assay.

Crystallization of the bacterial reaction center and determination of its X-ray structure has facilitated the identification of binding sites for all of the prosthetic groups attached to this complex (Deisenhofer et al., 1984, 1985; Yeates et al., 1988). By analogy with the bacterial reaction center, assignment of binding sites for most of the prosthetic groups of photosystem II (PS II)[1] has been attempted (Trebst, 1986; Michel & Deisenhofer, 1987). Additionally, the donors Z and D have been identified as Tyr 161 and Tyr 160 on D1 and D2, respectively (Debus et al., 1988a,b; Vermaas et al., 1988; Metz et al., 1989). The binding sites for functional Mn are an important exception since these have no counterparts in the bacterial system. Recently, models have been proposed suggesting that Mn binds to the D1 and D2 proteins (Coleman & Govindjee, 1987; Dismukes, 1988), and several reports support a location on D1 [Seibert & Cotton (1985), although D1 was called the "34-kDa protein"; Metz et al. (1986), Ikeuchi et al. (1988), Seibert et al. (1988, 1989), and Tamura et al. (1989)]. However, the exact nature of the ligands to Mn are largely unknown. EXAFS studies have indicated that N or O atoms are the most likely candidates for bridging and terminal ligands to Mn in PS II (Kirby et al., 1981; Yachandra et al., 1986; George et al., 1989). This led to the suggestion by Coleman and Govindjee (1987) that histidine, aspartate, and glutamate residues on the D1 and D2 proteins are possible contributors of terminal ligands to Mn. Similarly, Dismukes (1988) suggested aspartate and asparagine residues, and possibly also histidine, glutamate, and glutamine residues, on the same proteins as sources of ligands. Recent evidence supports the proposal that histidine residues, at least on the D1 protein (Tamura et al., 1989, Seibert et al., 1989), as well as carboxyl residues (Diner et al., 1990; Vermaas et al., 1990; Preston & Seibert, 1991) on D1 and D2 provide ligands to Mn.

Hsu et al. (1987) developed an assay for detecting a high-affinity Mn-binding site in PS II membranes. This assay depends on the observation that micromolar concentrations of Mn^{2+} can inhibit DPC electron donation to Mn-depleted

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[1] Abbreviations: Chl, chlorophyll; D, redox-active tyrosine 160 on the D2 reaction center protein; EDC, 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide hydrochloride; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; EXAFS, extended X-ray absorption fine structure, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDS–PAGE, lithium dodecyl sulfate–polyacrylamide gel electrophoresis; LF-1, low fluorescence mutant of *Scenedesmus obliquus*; MES, 4-morpholineethanesulfonic acid; PS II, photosystem II; RC, reaction center; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type; Z, redox-active tyrosine 161 on the D1 reaction center protein.

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PS II membranes noncompetitively (Preston & Seibert, 1991), and this has allowed for the examination of Mn-binding sites in greater detail. Seibert et al. (1989) used the assay to show that (1) the high-affinity Mn-binding site is present in isolated PS II reaction center complex [composed of D1, D2, 4.8-kDa, and cytochrome b_{559} proteins (Nanba & Satoh, 1987; Ikeuchi & Inoue, 1988a)], (2) treatment with the histidine chemical modifier DEPC inhibits half of the binding site in WT PS II membranes from *Scenedesmus obliquus* indicating that histidine provides half of the ligands to Mn, (3) half of the binding site (that affected by DEPC) is apparently missing in the O_2-evolution- and Mn-deficient LF-1 mutant of *Scenedesmus*, and (4) photoactivation requires the presence of all the high-affinity Mn-binding site. In this study, we have further investigated the high-affinity Mn-binding site purported to be the binding site for functional Mn [Hsu et al. (1987) and see also Preston and Seibert (1991)] using proteases to degrade lumenal surface protein segments of *Scenedesmus* PS II membranes. We have identified four separate components of a site involved in the high-affinity binding of Mn, of which two can be inhibited by the histidine-modifying reagent DEPC and two by the carboxyl-modifying reagent EDC. A preliminary report of this work is available (Preston & Seibert, 1990).

Experimental Procedures

S. obliquus wild-type and LF-1 cells were grown heterotrophically in 200-mL batches of NGY media (Bishop, 1971). Cells were harvested and broken, and PS II membrane fragments were prepared as described previously (Metz & Seibert, 1984). Spinach PS II membranes were prepared as reported before (Preston & Seibert, 1991). PS II membranes were frozen 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 use. Carboxypeptidase A type II from bovine pancreas (EC 3.4.17.1), subtilisin Carlsberg from *Bacillus subtilis* (EC 3.4.21.14), trypsin (TPCK treated) (EC 3.4.21.4), and the protease from *Staphylococcus aureus* strain V8 (EC 3.4.21.19) were purchased from Sigma (St. Louis, MO) and used as provided.

PS II membranes at 200 μg of Chl·mL^{-1} were incubated with the proteases at 20 °C in the dark in buffer B (20 mM MES-NaOH, pH 6.5, 0.4 M sucrose, and 20 mM NaCl), except for trypsin treatment where the buffer was 20 mM HEPES-NaOH, pH 7.4, 0.4 M sucrose, and 20 mM NaCl. Digestion conditions were as follows: 2:1 (w/w) carboxypeptidase A/Chl with a 1-h incubation, 1:20 (w/w) subtilisin/Chl with a 30-min incubation, 1:50 (w/w) trypsin/Chl with a 10-min incubation, and 50 units of V8 protease·(mg of Chl)$^{-1}$ with a 1-h incubation. With the exception of trypsin treatment, the conditions used for protease treatments were chosen to ensure that the reactions had gone to completion. The mild trypsin treatment conditions used in the current study were chosen on the basis of the work of (1) Jansson et al. (1979) who showed that a mild trypsin treatment at pH 7.4 attacks O_2-evolving capacity selectively and (2) Renger et al. (1984) who showed that a mild trypsin treatment for 10 min could specifically inhibit O_2 evolution of inside-out thylakoid membranes. Harsher treatments (not used in the current study) are known to affect the reducing side in addition to the oxidizing side of PS II. Phenylmethanesulfonyl fluoride (1 mM) was added to the carboxypeptidase A incubation buffer to inhibit endopeptidase activity. The proteases were inhibited at the end of the treatments by adding 20 volumes of ice-cold buffer B (for V8 protease), buffer B containing 4 mM 1,10-phenanthroline (for carboxypeptidase A treatment), or buffer B containing 1 mM phenylmethanesulfonyl fluoride (for subtilisin and trypsin treatments). The membranes were collected by centrifugation (30000*g*, 10 min), washed once in buffer B, and resuspended in buffer A.

Mn depletion of PS II membranes was accomplished either by NH_2OH treatment (which does not remove the extrinsic proteins at pH 6.5) or Tris treatment (which removes the extrinsic proteins). PS II membranes were incubated at 0.5 mg of Chl·mL^{-1} with either 5 mM NH_2OH in buffer A, or 0.8 M Tris-HCl, pH 8.4 (or 1.0 M Tris-HCl, pH 9.4, for spinach) and 0.4 M sucrose for 30 min at 4 °C. The membranes were collected by centrifugation (30000*g*, 10 min), washed once, and resuspended in buffer A. Histidine residues were modified with 500 μM DEPC (Tamura et al., 1989; Seibert et al., 1989), and carboxyl residues were modified with 10 mM EDC as described in the accompanying paper (Preston & Seibert, 1991). All chemical modifications were performed on Mn-depleted PS II membrane preparations. Specificity of the two chemical modifiers under the conditions described has been addressed elsewhere (Tamura et al., 1989; Preston & Seibert, 1991). We also show in the accompanying paper (Preston & Seibert, 1991) that EDC and DEPC effects on the high-affinity Mn-binding site are mutually exclusive. For this reason, and those outlined below, we need show the effects of only one chemical modifier in the figures. Where we have data on the other chemical modifier, we have indicated this in the text. We did not perform EDC modifications on NH_2OH-treated PS II membranes since we found that EDC cross-links the extrinsic polypeptides. This appears to interfere with the assays, causing spurious results. In general we also like to avoid using DEPC more than necessary because of its toxicity. The order of treatments for each sample is indicated in the figure legends.

Initial rates of DCIP photoreduction were measured with an Aminco DW2a spectrophotometer in the split beam mode as described in the accompanying paper (Preston & Seibert, 1991). Manganese analyses were performed with a Thermo Jarrell Ash ICAP-61 inductively coupled argon plasma spectrometer, Thermospec version 4.1 software, and EPA method 200.7 (Environmental Protection Agency, 1983). This method involves hydrolyzing the organics with 50% nitric acid, evaporating the resulting solution to 20% volume, doubling the volume with concentrated hydrochloric acid, heating briefly to hydrolyze any surviving organic materials, and bringing the solution back to a predetermined volume with deionized water for Mn analysis.

Results

The assay system of Hsu et al. (1987) can be used to determine the amount of high-affinity Mn-binding site (Seibert et al., 1989; Preston & Seibert, 1989, 1991) present in different preparations of PS II membrane fragments from which Mn has been extracted. At the concentrations of DPC, $MgCl_2$, and NaCl used in our studies, ≥5 μM $MnCl_2$ inhibits DPC-supported DCIP photoreduction by about 50% in WT material. This can be seen for *Scenedesmus* WT PS II membrane fragments in Figure 1, and we will demonstrate that this inhibition corresponds to the presence of four independent components of the high-affinity Mn-binding site. Treatment of these membranes with a high concentration of carboxypeptidase A decreased the amount of $MnCl_2$ inhibition of DPC → DCIP activity. In this case we observed about 37% inhibition of activity at 10 μM $MnCl_2$. Increasing the $MnCl_2$ concentration to 100 μM did not increase further the inhibition of activity. A similar situation was apparent when PS II membranes were treated with a high concentration of subtilisin. DCIP photoreduction was inhibited by 38% in the presence

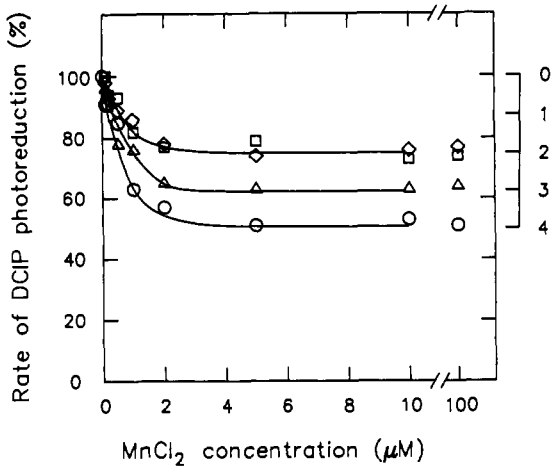

FIGURE 1: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in carboxypeptidase A treated *S. obliquus* WT PS II membranes. PS II membranes were treated with NH_2OH (○); carboxypeptidase A then NH_2OH (△); NH_2OH then DEPC (□); or carboxypeptidase A, NH_2OH, and then DEPC (◇). Maximum rates of DCIP photoreduction were 118, 147, 109, and 134 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for NH_2OH-treated, carboxypeptidase A treated, DEPC-treated, and carboxypeptidase A plus DEPC-treated PS II membranes, respectively. The scale to the right in this and subsequent figures represents the number of high-affinity Mn-binding sites detectable in the sample. Each "site" may represent an individual ligand available for binding Mn (see Discussion).

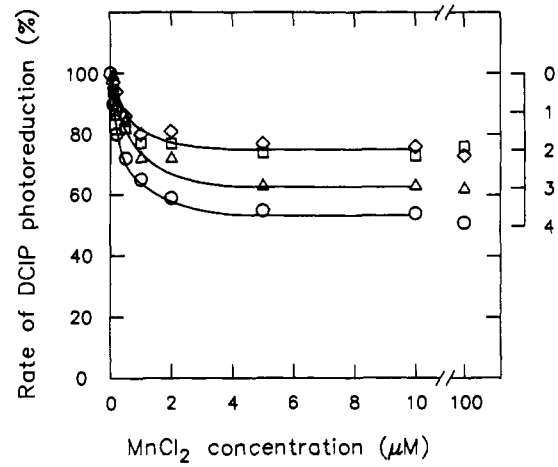

FIGURE 2: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in subtilisin-treated WT PS II membranes. PS II membranes were treated with NH_2OH (○); subtilisin then NH_2OH (△); NH_2OH then DEPC (□); or subtilisin, NH_2OH, and then DEPC (◇). Maximum rates of DCIP photoreduction were 119, 126, 109, and 112 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for control, subtilisin-treated, DEPC-treated, and subtilisin- plus DEPC-treated PS II membranes, respectively.

of 10 μM $MnCl_2$ after treatment compared to a 49% inhibition prior to treatment (Figure 2). If a 50% inhibition of DPC → DCIP activity by $MnCl_2$ is indicative of the complete high-affinity Mn-binding site, then the amount of inhibition seen after the protease treatments (37–38%) corresponds to about three-fourths of the site remaining. Thus both of these protease treatments appear to be degrading one-fourth of the high-affinity Mn-binding site. Previously (Seibert et al. 1989; Preston & Seibert 1989, 1991), we were able to divide the high-affinity Mn-binding site into two components. The data provided in this section suggest that the site contains at least four independent components. Consequently, on the right-hand side of the figures, we indicate the number of high-affinity Mn-binding sites remaining after treatment, assuming that there are a total of four possible sites (see Discussion).

It has been shown previously that the histidine chemical modifier DEPC can inhibit half of the high-affinity Mn-

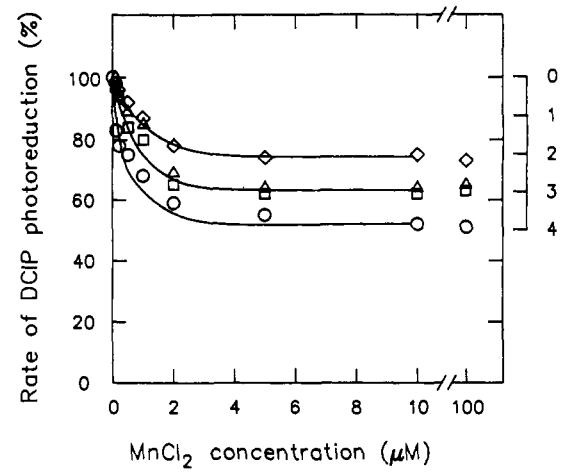

FIGURE 3: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in carboxypeptidase A and subtilisin-treated WT PS II membranes. PS II membranes were treated with NH_2OH (○); carboxypeptidase A then NH_2OH (△); subtilisin then NH_2OH (□); or carboxypeptidase A, subtilisin, and then NH_2OH (◇). Maximum rates of DCIP photoreduction were 121, 154, 117, and 140 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for NH_2OH-treated, carboxypeptidase A treated, subtilisin-treated, and carboxypeptidase A plus subtilisin-treated PS II membranes, respectively.

binding sites (Seibert et al., 1989). Protease-treated PS II membranes were subsequently modified with DEPC to determine which (i.e., DEPC-sensitive or DEPC-insensitive) of the high-affinity Mn-binding sites are affected by the protease treatments. DEPC reduced $MnCl_2$ inhibition of DPC → DPIC activity from about 37% to about 25% for PS II membranes treated with either protease. DEPC also reduced the $MnCl_2$ inhibition of control PS II membranes to about 25% from about 50% (Figures 1 and 2). This indicates that both protease treatments are affecting a DEPC-sensitive binding site. However, if these two protease treatments each affect one of two DEPC-sensitive high-affinity Mn-binding sites, the possibility arises that they could be acting on the same site. We tested this possibility in the following experiment. PS II membranes were treated sequentially with the two proteases, first with carboxypeptidase A and then with subtilisin. The PS II membranes were treated in this order to limit carboxypeptidase A action to the true carboxyl ends of proteins and not to new segments produced by subtilisin action. The results of this experiment are shown in Figure 3. $MnCl_2$ inhibited NH_2OH-treated PS II membrane activity by 50%, carboxypeptidase A- or subtilisin-treated PS II membranes by 36–39%, and PS II membranes treated with both carboxypeptidase A and subtilisin by 24%. This demonstrates that carboxypeptidase A and subtilisin are affecting separate DEPC-sensitive high-affinity Mn-binding sites. It should also be observed that the extrinsic proteins do not interfere with DEPC modification of the high-affinity Mn-binding sites since the results obtained here with NH_2OH-treated PS II membranes are identical with those obtained with Tris-treated PS II membranes (Preston & Seibert, 1991). We also have performed EDC modification of Tris-treated PS II membranes after treatment of these membranes with either subtilisin or carboxypeptidase A. In both cases $MnCl_2$ could only inhibit DPC → DCIP activity by 11–13% (data not shown), the expected result for mutually exclusive protease and EDC action. This confirms our interpretation that both carboxypeptidase A and subtilisin affect DEPC-sensitive high-affinity Mn-binding sites.

Modification of Tris-treated PS II membranes with trypsin resulted in the degradation of two high-affinity Mn-binding sites (Figure 4A). In this case, 10 μM $MnCl_2$ inhibited DPC

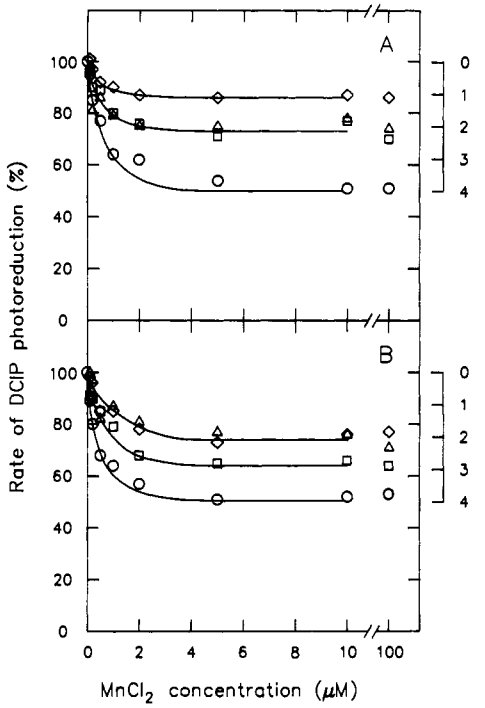

FIGURE 4: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in trypsin-treated WT PS II membranes. (A) Control PS II membranes were Tris-treated (○). These membranes were then treated with trypsin (□), EDC (△), or trypsin then EDC (◇). Maximum rates of DCIP photoreduction were 151, 134, 102 and 105 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated, EDC-treated, trypsin-treated, and trypsin- then EDC-treated PS II membranes, respectively. (B) Control PS II membranes were NH_2OH treated (○). These membranes were then treated with either trypsin (□), DEPC (△), or trypsin then DEPC (◇). Maximum rates of DCIP photoreduction were 162, 130, 139, and 133 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for NH_2OH-treated, DEPC-treated, trypsin-treated, and trypsin- then DEPC-treated PS II membranes, respectively.

→ DCIP activity by 23%. Further treatment of Tris- and trypsin-treated WT PS II membranes with the carboxyl-modifying reagent EDC decreased Mn^{2+} inhibition of DPC → DCIP activity from 25% to 13%, leaving only one high-affinity Mn-binding site available (Figure 4A). We show in the accompanying paper that EDC blocks that half of the high-affinity Mn-binding sites not affected by DEPC (Preston & Seibert, 1991), and therefore trypsin must be degrading two Mn-binding sites, one that is DEPC-sensitive and a second that is EDC-sensitive.

In contrast to the situation observed with Tris-treated PS II membranes, trypsin modification of NH_2OH-treated PS II membranes (Figure 4B) resulted in the degradation of fewer high-affinity Mn-binding sites, i.e., trypsin degraded only one of the binding sites in NH_2OH-treated material. When NH_2OH- and trypsin-treated PS II membranes were further treated with DEPC, $MnCl_2$ inhibition of DPC → DCIP activity decreased from 37% to 25% (Figure 4B). Since DEPC alone decreases activity to 25%, trypsin must degrade the DEPC-sensitive binding site but not the EDC-sensitive site in NH_2OH-treated PS II membranes. Due to problems associated with EDC modification of PS II membranes containing the extrinsic polypeptides as outlined under Experimental Procedures, we used DEPC instead of EDC in Figure 4B. The different results obtained from trypsin treatment of Tris- compared to NH_2OH-treated PS II membranes stem from the fact that Tris treatment removes the extrinsic proteins as well as the functional Mn, whereas NH_2OH treatment at the pH we used removes only the Mn [Preston and Seibert, unpublished observation; see also Tamura and Cheniae (1987)]. We

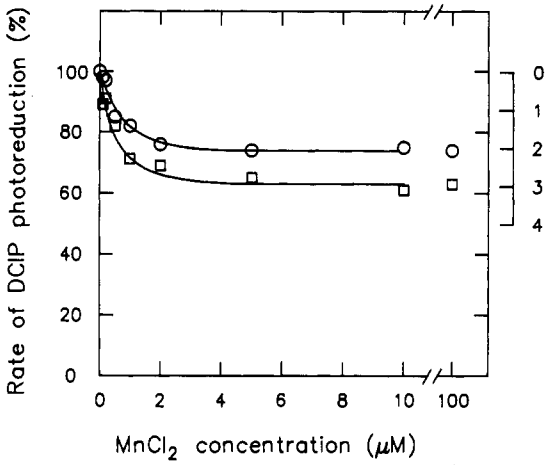

FIGURE 5: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in trypsin-treated *Scenedesmus* LF-1 PS II membranes. Control PS II membranes (○); and PS II membranes treated with trypsin (□). Maximum rates of DCIP photoreduction were 144 and 118 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for control and trypsin-treated PS II membranes, respectively.

also performed trypsin treatment on *Scenedesmus* LF-1 PS II membranes, which bind the 30-kDa protein but bind neither the 20- nor 16-kDa extrinsic proteins (Metz et al., 1985). Figure 5 shows that inhibition by 10 μM $MnCl_2$ of DPC → DCIP photoreduction in LF-1 PS II membranes[2] increases from 25 to 37% after trypsin treatment. Half of the high-affinity Mn-binding site appears to be absent in LF-1 PS II membranes (Seibert et al., 1989), and we have previously shown (Preston & Seibert, 1989) that low concentrations of proteases can restore the full complement of high-affinity Mn-binding sites in LF-1 PS II membranes (the DEPC-sensitive sites are the ones restored) by removing a few amino acids from the carboxyl end of the unprocessed D1 protein. On the basis of these observations, we interpret the results of trypsin degradation of LF-1 PS II membranes in the following way. Trypsin cleaves the carboxyl end of the LF-1 D1 protein, thereby exposing the two shielded binding sites. In addition, it removes one of the trypsin-sensitive high-affinity Mn-binding sites. The other trypsin-sensitive Mn-binding site (that observed in Figure 4A) is not affected. This second site appears therefore to be protected from protease action by the 30-kDa protein. Thus, these experiments indicate that the 30-kDa protein protects one of the EDC-sensitive Mn-binding sites from degradation by trypsin.

We further investigated the trypsin-sensitive high-affinity Mn-binding sites using other proteases. As seen above, the trypsin-sensitive component comprises two sites, one sensitive to the histidine modifier DEPC and the other sensitive to the carboxyl modifier EDC. Modification of Tris-treated WT PS II membranes first with carboxypeptidase A and then with trypsin yields the same result as treatment with trypsin alone, namely $MnCl_2$ inhibition of DPC → DCIP activity is reduced from about 50% to about 25% (Figure 6). This indicates that the DEPC-sensitive binding site that is degraded by trypsin is the same as the carboxypeptidase A sensitive binding site. In contrast to the situation observed with the trypsin experiments, removal of the extrinsic proteins does not increase the number of high-affinity Mn-binding sites affected by either carboxypeptidase A treatment (compare Figure 1 with Figure 6) or subtilisin treatment (data not shown). We also attempted

[2] Previous studies showed that LF-1 and NH_2OH-treated LF-1 membranes are indistinguishable by the DPC/Mn^{2+} assay (Seibert et al., 1989).

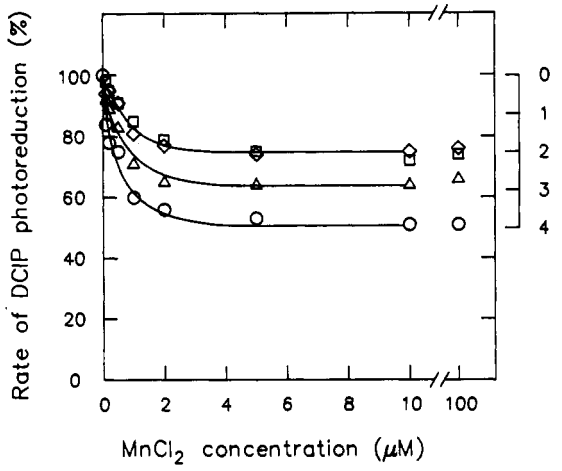

FIGURE 6: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in carboxypeptidase A and trypsin-treated WT PS II membranes. Control PS II membranes were Tris treated (○). These membranes were then treated with either carboxypeptidase A (△), trypsin (□), or carboxypeptidase A then trypsin (◇). Maximum rates of DCIP photoreduction were 121, 155, 110, and 145 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated, carboxypeptidase A treated, trypsin-treated, and carboxypeptidase A then trypsin-treated PS II membranes, respectively.

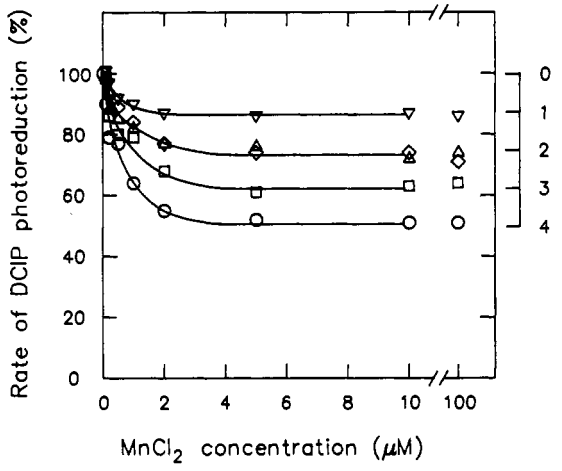

FIGURE 7: $MnCl_2$ inhibition of DPC-supported DCIP photoreduction in V8 protease treated WT PS II membranes. Control PS II membranes were Tris-treated (○). These membranes were then treated with either V8 protease (□), trypsin (△), V8 protease then trypsin (◇), or V8 protease then EDC (▽). Maximum rates of DCIP photoreduction were 138, 110, 115, 104, and 100 μmol of DCIP·(mg of Chl)$^{-1}$·h^{-1} for Tris-treated, V8 protease-treated, trypsin-treated, V8 protease then trypsin-treated, and V8 protease then EDC-treated PS II membranes, respectively.

to degrade Mn-binding sites using V8 protease, which under the conditions we used is probably specific for the carboxyl side of glutamate residues (Houmard & Drapeau, 1972) but may also cleave at aspartate residues. This protease was chosen in an attempt to remove one or both of the EDC-sensitive Mn-binding sites, which we know involve carboxyl residues (Preston & Seibert, 1991). Following extensive V8 protease treatment of WT PS II membranes, $MnCl_2$ inhibition of DPC → DCIP activity decreased from 49% to 37% (Figure 7), suggesting that the enzyme degrades one binding site. Additional modification of V8 protease-treated PS II membranes with EDC further reduced the $MnCl_2$ inhibition of DPC → DCIP activity to 12%. Surprisingly, V8 protease appears to degrade a histidine site instead of a carboxyl site. Sequential treatment of PS II membranes with V8 protease followed by trypsin reduced the $MnCl_2$ inhibition of DPC → DCIP activity to 24%. This is the same result as obtained after treatment

Table I: Effects of Protease Treatments of *Scenedesmus* Thylakoid Membranes on the High-Affinity Mn-Binding Site Compared to Those of Treated 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	0.49
carboxypeptidase A PS II	0.65
carboxypeptidase A thylakoids/PS II	0.47
subtilisin PS II	0.62
subtilisin thylakoids/PS II	0.49
V8 protease PS II	0.65
V8 protease thylakoids/PS II	0.50

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

with trypsin alone, suggesting that the V8-sensitive site is the same as one of the trypsin-sensitive sites. We have now established that the V8 protease-sensitive high-affinity Mn-binding site is not EDC-sensitive but is the same as one of the trypsin-sensitive sites. On the basis of these results, we suggest that the V8-sensitive Mn-binding site is probably identical with the carboxypeptidase A sensitive Mn-binding site.

To determine whether the action of the proteases on the high-affinity Mn-binding site is due to cleavage on the stromal or lumenal side of the membrane, we first performed the protease digestions on thylakoid membranes (stromal side exposed and lumenal side sequestered and unavailable for protease action). The thylakoid membranes were then fractionated into PS II membrane fragments (lumenal side exposed), the Mn was removed by Tris treatment, and finally the membranes were assayed for DPC-supported DCIP photoreduction. Control PS II membrane fragments were exposed to similar protease treatments and assayed in parallel experiments. The data are presented in Table I as the fraction of activity remaining in the presence of 10 μM $MnCl_2$ compared to activity in the absence of $MnCl_2$. These experiments suggest that, at least for carboxypeptidase A, subtilisin, and V8 protease, protease action on the lumenal side of the membrane is required to affect the high-affinity Mn-binding sites. The same experiment could not be performed with trypsin since trypsin treatment of thylakoid membranes cleaves LHCII (Steinback et al., 1979), inhibiting membrane stacking and consequently our ability to obtain PS II membranes. Since we were unable to obtain PS II membranes after trypsin treatment, trypsin results are not reported in Table I. Nevertheless, we have previously established that one trypsin site can be degraded by both carboxypeptidase A and V8 protease and the other is inhibited by EDC. Thus, from the results of Table I, we have no reason to suspect that trypsin would act differently from the other proteases. In addition, we have shown in the accompanying paper (Preston & Seibert, 1991) that all of the high-affinity Mn-binding sites can be modified with either DEPC or EDC, both of which act on the lumenal side of the membrane.

The result of chemical modifier and protease treatments on Mn-depleted PS II membrane fragments as probed by the DPC/Mn^{2+} inhibition assay demonstrates that the high-affinity Mn-binding site is composed of four components. The salient question now is whether the "four components" (or "sites" as we have termed them) correspond to four ligands binding one Mn or four ligands binding two or more Mn. In

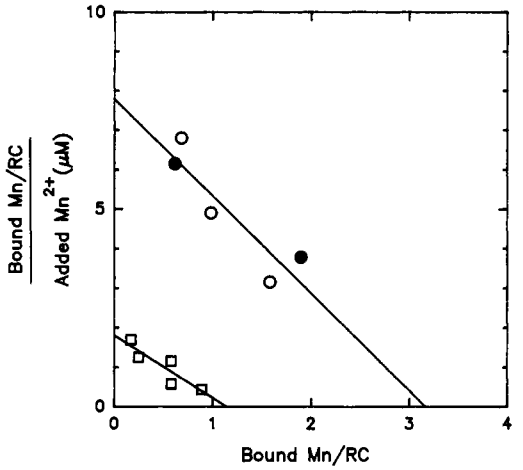

FIGURE 8: Eadie–Hofstee plots of Mn bound to Tris-treated spinach PS II membranes exposed to the DPC/Mn^{2+} assay conditions. Excess free Mn was removed, and the membranes were collected for metal analysis by the centrifugation (□) or dialysis/evaporation (○ and ● denote different experiments) methods described in the text. The membranes were not treated with chemical modifiers or proteases.

order to address this question (see Discussion also), we directly measured the amount of Mn binding to the membrane under the actual assay conditions. Figure 8 is an Eadie–Hofstee plot of the results of these measurements, which were carried out in two ways. Initially, Tris-treated PS II membrane fragments were reacted at 5 μg of Chl/mL, with 200 μM DPC, 30 μM DCIP, 0.4 M sucrose, 20 mM NaCl, 50 mM MES/NaOH (pH 6.5), and from 0.1 to 10 μM $MnCl_2$ in the dark and then exposed to saturating light for several seconds. In the first case, the membranes were centrifuged in the dark. The pellets were resuspended in minimal glass-distilled water and assayed for Chl and Mn. The results in this case was that about 1 Mn per RC was bound with a K_M of around 0.6 μM. In the second case, the treated membranes were dialyzed three times in the dark against the above treatment buffer (without membranes and $MnCl_2$) to decrease the amount of residual free Mn and then evaporated in clean pyrex beakers down to a manageable volume for Mn assays. However, even after dialysis, free Mn still remained in the samples. Consequently, before we could generate the Eadie–Hofstee plot in Figure 8 (circles) from the apparent Mn/RC vs added Mn^{2+} curve, we had to subtract out the free Mn part of the apparent Mn/RC value measured in order to obtain a bound Mn/RC vs added Mn^{2+} curve. We did this by calculating observed $Mn{\cdot}RC^{-1}{\cdot}(\text{added } Mn^{2+})^{-1}$ values for three to four samples to which 1–5 μM Mn^{2+} was added originally. In this range the $Mn{\cdot}RC^{-1}{\cdot}(\text{added } Mn^{2+})^{-1}$ values ceased decreasing. Using the average for the $Mn{\cdot}RC^{-1}{\cdot}(\text{added } Mn^{2+})^{-1}$ values determined over this range of added Mn^{2+}, we were able to extrapolate and then subtract out the free Mn component from the apparent Mn/RC vs added Mn^{2+} curve at low added Mn^{2+} values in order to obtain the Eadie–Hofstee plot for bound Mn. The result was about 3.2 Mn bound per RC with a K_M of around 0.4 μM. We recognized that there might be uncertainties about the above mentioned subtraction at added Mn^{2+} values in the 0.1–0.5 μM range where we obtained the data for Figure 8 (circles). Consequently, we attempted to vary the estimated free Mn concentration at these low values of added Mn^{2+}. While this variation did affect the calculated K_M value, the influence on the number of bound Mn/RC detected was minor.

Discussion

Degradation of protein segments from *Scenedesmus* PS II membrane fragments with proteases has allowed us to identify a minimum of four independent high-affinity Mn-binding sites (Figures 3 and 4) located on the lumenal side of the membrane (Table I). Each of these sites contributes equally (Figure 7) to the noncompetitive inhibition by $MnCl_2$ of DPC-supported DCIP photoreduction [see Preston and Seibert (1991)]. Two of the sites are sensitive to the histidine chemical modifier DEPC, and the other two are sensitive to the carboxyl chemical modifier EDC. Figure 8 shows that under the assay conditions more than three Mn per reaction center are still bound to the membrane (after about 3 h of dialysis) with a K_M of around 0.4 μM if care is taken to prevent Mn from dissociating from the membrane during preparation for metal analysis.[3] The Mn stoichiometry that we measure directly is consistent with the four high-affinity sites that we predict from the DCP/Mn^{2+} assay each binding one Mn. Furthermore, the K_M that we estimate is close to the Mn dissociation constants that we have measured previously in Mn-depleted *Scenedesmus* PS II membranes (Seibert et al., 1989).[4] As stated in the accompanying paper (Preston & Seibert, 1991), Mn appears to prevent DPC donation to one of two intrinsic PS II donors (probably Z; D. J. Blubaugh and G. M. Cheniae, personal communication) at the high DPC concentrations used in the assay, but details of the interaction remain to be determined. Although the four high-affinity sites correlate well with the four Mn per reaction center known to be involved in O_2 evolution [see Amesz (1983) and Dismukes (1986) for reviews], the membranes do not evolve O_2 under the conditions of the DPC/Mn^{2+} assay (data not shown). Thus, the relationship between these sites, those described in the normal photoactivation process (Tamura & Cheniae, 1987), and those that bind Mn in the functional form are not clear at this point. We do know, however, that one high-affinity Mn-binding site is present (in the absence of DPC) at the beginning of the normal photoligation process, and three others are formed during photoligation (Tamura & Cheniae, 1987; Miller & Brudvig, 1990). The presence of high DPC concentration may allow for the formation and loading of all four sites concurrently, albeit in a nonfunctional conformation (i.e., the bridging ligands required for an active tetrameric Mn complex may not form properly). The fact that DEPC modifies histidines required both for photoligation (Tamura et al., 1989) as well as for binding high-affinity Mn (Seibert et al., 1989; Preston & Seibert, 1991) supports this assertion.

[3] Figure 8 (squares) shows that if care is not taken to maintain the normal ratio of DPC to Chl required in the DPC/Mn^{2+} assay while free Mn is removed and the membranes collected for metal analysis, only one high affinity Mn per reaction center apparently remains on the membrane. In the accompanying paper (Preston & Seibert, 1991), we demonstrate that Mn inhibits DCIP photoreduction by DPC only in the high DPC concentration range (Appendix, Figure 8) where we observe four high-affinity Mn-binding sites. At zero or low DPC concentration, Mn^{2+} acts as a donor to PS II and only one high-affinity site is observed [see also Hoganson et al. (1989)]. Apparently, if membranes are centrifuged to remove free Mn, the effective DPC to Chl ratio decreases, shifting the membranes from the condition where Mn acts as an inhibitor to that where Mn^{2+} acts as a donor and all but one bound Mn are lost.

[4] In actuality the situation could be more complex and neither of the assays for Mn binding may give a measurement of the true dissociation constant or K_M for Mn^{2+}. The samples, exposed to preillumination, generate some bound Mn^{3+}, which may be the species that blocks DPC donation to PS II as discussed in the accompanying paper (Preston & Seibert, 1991). Thus, it is likely that the Mn remaining bound to the PS II membrane fragments in Figure 8 (circles) is controlled by a combination of at least three factors: (1) the dissociation constant(s) for Mn^{2+}, (2) the rate of Mn^{3+} formed during illumination, which may be active in preventing DPC donation, and (3) the stability of the bound photochemically generated Mn^{3+} on the membrane. The fact that we measure closer to three rather than four Mn per reaction center in Figure 8 may be an indication of item 3 above.

Considering the complexity of the DPC/Mn^{2+} assay and the metal analysis studies, an alternative possibility should be discussed. The four high-affinity "sites" that we can detect by DEPC, EDC, and protease treatments may in fact reflect four ligands to a single Mn rather than four ligands with each binding one of four Mn. In this case, the effects of EDC, DEPC, and protease treatments could arise from changes in the rate of Mn^{3+} production, stability of bound Mn, and/or accessibility of the bound Mn in shutting off DPC donation. However, in light of both the Mn metal analyses results and the fact that two high-affinity sites become detectable when only two of four functional Mn are removed from the membrane (Preston & Seibert, 1991), this interpretation seems less likely. Nevertheless, we can not completely eliminate the possibility that the four detectable ligands are associated with one (perhaps the first) Mn bound in the photoligation/photoactivation process and that the other Mn, bound when high DPC concentration are present, do not affect DPC donation. This notwithstanding, we can make some specific observations about some of the high-affinity sites.

The DEPC- and carboxypeptidase-A-sensitive Mn-binding site appears well exposed to protease action since all of the enzymes used, with the exception of subtilisin (which degrades the other DEPC-sensitive site), were able to degrade this site. This was a little surprising since subtilisin is the least specific of the proteases used, perferring large aromatic residues or, less commonly, aliphatic residues (Kraut, 1977). There are several such residues on the carboxyl end of the D1 protein, and we know that subtilisin cleaves a small part of the carboxyl end of this protein in both LF-1 and WT PS II membranes (Preston & Seibert, 1989). In contrast to both of the DEPC-sensitive Mn-binding sites, the EDC-sensitive sites are more protected. The EDC- and trypsin-sensitive Mn-binding site is protected from protease action by the 30-kDa extrinsic protein (Figures 4 and 5), which suggests that this protein is closely associated with reaction center proteins. We have not as yet been able to degrade the fourth binding site using the proteases so far examined, suggesting that this site is also well protected.

The experiments reported here in conjunction with the known specificities of the proteases used provide some clues as to possible lumenal-side locations of the high-affinity Mn-binding sites associated with histidyl and carboxyl residues. At present we know that the high-affinity Mn-binding sites (though not necessarily all sources of terminal ligands to Mn) are all present in the isolated PS II reaction center preparation (Seibert et al. 1989), which narrows down the number of possible proteins able to contribute ligands to five: D1, D2, the two subunits of cytochrome b_{559} (Nanba & Satoh, 1987), and the 4.8-kDa protein (Ikeuchi & Inoue, 1988a). Additionally, the D1 protein has been implicated as a possible binding site for Mn (Metz et al,. 1986; Ikeuchi et al., 1988; Seibert et al., 1988; 1989; Tamura et al., 1989; Preston & Seibert, 1989). Since none of the small proteins (cytochrome b_{559} or 4.8-kDa proteins) has histidine residues on the lumenal C-terminal end (Tae et al., 1988; Vallon et al., 1989; Ikeuchi & Inoue, 1988b; Webber et al., 1988), we can eliminate these proteins from the discussion concerning histidine sites.

As mentioned previously, there are two independent DEPC-sensitive Mn-binding sites, one affected by both carboxypeptidase A and V8 protease (Figures 1 and 7) and the other by subtilisin (Figure 2). Each of these sites involves a different histidine residue (Figure 3). The spinach D1 and D2 proteins contain a total of eight histidine residues on the lumenal side of the membrane (Trebst, 1986; see also Figure 9). Of these residues, His 190 (D1) and His 190 (D2) will not be further considered since they are probably too close to the membrane to be affected by the proteases. There is also the suggestion (Dismukes, 1988) that these residues are involved in binding other prosthetic groups. The number of histidine residues available to bind Mn can be reduced further by noting the results of Tamura et al. (1989) who on the basis of [^{14}C]DEPC experiments suggested that at least one histidine which provides a ligand to functional Mn is located on the D1 protein. They could find no evidence for histidines on D2 being involved in Mn-binding, although the possibility has not as yet been eliminated. Of the protease-sensitive binding sites, we know the carboxypeptidase A sensitive histidine site must be located on the carboxyl end of one of the reaction center proteins, probably the D1 protein. Carboxypeptidase A removes amino acids from the carboxyl end of proteins sequentially and does not usually release proline, hydroxyproline, or arginine residues (Ambler, 1967). Previously (Preston & Seibert, 1989) we have shown that a low concentration of carboxypeptidase A [1:20 (w/w) enzyme/Chl] removes several amino acids from WT D1 without affecting the high-affinity Mn-binding site. The carboxyl end of the processed spinach D1 protein has been identified as Ala 344 (Takahashi et al., 1988). Assuming a high degree of homology with *Scenedesmus* D1, this suggests that the low concentration of carboxypeptidase A probably removes four amino acids from the processed D1 protein stopping at Pro 340. This is consistent with removal of less than 1 kDa from this protein as seen with LDS–PAGE (Preston & Seibert, 1989). The higher concentration of carboxypeptidase A used in the current study must remove more of the D1 or D2 proteins because in this case one of the high-affinity Mn-binding sites is also affected. Assuming that D1 is affected, carboxypeptidase would have to digest through Pro 340, and we have LDS–PAGE evidence (data not shown) suggesting that this is occurring. The next resistant amino acid on D1 is Arg 334, which is 11 amino acids from the carboxyl end. If this is the termination point for carboxypeptidase A action under our conditions, then His 337 is implicated in the binding of Mn. Site-directed mutants of His 337 (to Leu, Asn, Gln, or Tyr) on D1 to not exhibit photoautotrophic growth on plates (R. J. Debus, personal communication), consistent with our results. His 337 on D2

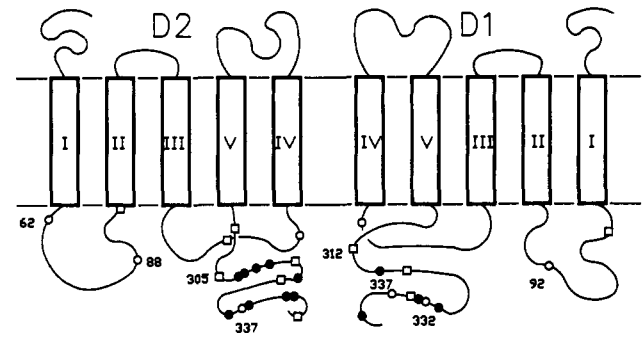

FIGURE 9: Diagram of the D1 and D2 proteins modeled after Trebst (1986). The open circles represent the approximate positions of histidine residues and squares of arginine and lysine residues. Only those residues on the lumenal side of the membrane are marked. The carboxypeptidase A sensitive binding site is suggested to be His 337 (D1). Arg 312 (D1) and Arg 305 (D2) are the possible trypsin cleavage points on the D1 and D2 proteins, respectively, that would remove the most protein. The carboxyl residues (Asp and Glu) that would be removed by these cuts and that could be high-affinity Mn-binding sites are indicated by closed circles. Note that trypsin cannot remove segments of any of the loops from the lumenal side of the membrane since no loop contains more than one trypsin cleavage point.

is unlikely to be the carboxypeptidase A sensitive high-affinity Mn-binding site because there is an arginine residue at 349 on D2 (Holshuh et al., 1984), which would inhibit carboxypeptidase A action beyond this point. We believe, on the basis of the above arguments, that His 337 in D1 is the most likely site for the carboxypeptidase A sensitive high-affinity Mn-binding site; however, sequencing the remaining carboxyl ends of D1 and D2 after carboxypeptidase A treatment must be done to unambiguously rule out either His 332 on D1 or His 337 on D2. His 332 on D1 may supply a ligand to the functional tetrameric Mn complex on the basis of site-directed mutant studies (Nixon & Diner, 1991) and could provide a ligand to high-affinity Mn not detectable by the DPC/Mn^{2+} assay.

Trypsin degrades two high-affinity Mn-binding sites, one of which is DEPC sensitive and is the same site degraded by carboxypeptidase A (Figure 6). This requires trypsin cleavage of the D1 protein prior to His 337. Trypsin cleaves at the carboxyl side of arginine or lysine residues, but we need not consider lysine residues on D1 since the D1 protein of *Scenedesmus* contains only one lysine that is not near the carboxyl end (Metz et al., 1985). However, there are three arginines on the carboxyl end of the D1 protein (residues 312, 323, and 334) and cleavage at any one of these would lead to removal of the putative carboxypeptidase A sensitive binding site (see Figure 9). Thus, our identification of His 337 on D1 as a high-affinity site is consistent with the known sequence of D1 and the expected action of both proteases.

The subtilisin-sensitive site is also DEPC sensitive and is presumably a histidine residue. This site may be His 92 on D1 or any of His 62, His 88, or His 337 on D2 (Figure 9). His 332 on D1 is unlikely to be the subtilisin-sensitive Mn-binding site since a cleavage act removing this residue would also remove His 337 (which we suggest is the carboxypeptidase A sensitive site). His 92 on D1 may not be the site because mutations at this residue do not inhibit photosynthetic growth (Debus et al., 1991). His 337 on D2 is attractive on the basis of asymmetry arguments, and, as expected of the subtilisin site (Figure 2), it is likely to be in a position exposed to enzymatic action.

There are also two EDC-sensitive Mn-binding sites present in PS II as can be observed from the trypsin experiments (Figure 4). We can gain a little information on the possible location of the EDC- and trypsin-sensitive binding site from the protein folding model of the D1 and D2 proteins proposed by Trebst (1986). If we assume that trypsin is degrading the Mn-binding site by removing that segment of protein containing the site, then the binding site is probably not located on the lumenal loops of either the D1 or D2 proteins since these segments of protein cannot be removed by trypsin (Figure 9). There are, however, several carboxyl residues on the carboxyl ends of D1 and D2, four on D1 and nine on D2, which could potentially be removed by trypsin. The fact that the 30-kDa extrinsic protein protects the site from trypsin suggests that it is not located at the terminus of the carboxyl end of D1 or D2. Aspartate 242 and the carboxyl end of D1 (Ala 344), for example, can also be eliminated on the basis of the carboxypeptidase work. On the other hand, these residues may contribute ligands to the functional Mn complex (Debus et al., 1991; Nixon & Diner, 1991) or a ligand to high-affinity Mn that is not detectable by the DPC/Mn^{2+} assay. A location on the carboxyl end of the cytochrome b_{559} or 4.8-kDa proteins for EDC-sensitive sites is also possible since there are several carboxyl residues on these segments (Ikeuchi & Inoue, 1988b; Tae et al., 1988; Vallon et al., 1989) and trypsin cleavage has been observed (Tae & Cramer, 1989). If our segment removal assumption is not correct, Asp 170 on D1 (Diner et al., 1990; Debus et al., 1991), Glu 69 on D2 (Vermaas et al., 1990), and Asp 61 or Glu 65 on D1 (Debus et al., 1991), all of which have been proposed from site-directed mutant studies as supplying ligands to functional Mn, may also be candidates for either of the two EDC-sensitive high-affinity Mn-binding sites.

An alternative to direct protease (or amino acid modifier) action on putative Mn-binding sites could be that the proteases are acting at a site remote from the direct point of Mn binding and that allosteric effects are leading to an apparent loss of the Mn-binding site. Our previous results as discussed in the accompanying paper (Preston & Seibert, 1991) indicate that this possibility is not likely. We also point out that four such allosteric effects must be postulated, one for each high-affinity Mn-binding sites.

In the foregoing discussion it should be remembered that each Mn (either high affinity or functional) in PS II may have as many as two to four terminal ligands to amino acids (Kirby et al., 1981; Kambara & Govindjee, 1985; Yachandra et al., 1986; Brudvig & Crabtree, 1986; Coleman & Govindjee, 1987; Dismukes, 1988). Therefore, it is possible that residues providing ligands to Mn other than histidines or carboxyls detectable directly by the DPC/Mn^{2+} assay may actually be the ones affected by the protease treatments while still effectively inhibiting either the "histidine" or "carboxyl" sites. There may also be other ligands to Mn that our assay can not detect [see Preston and Seibert (1991)] and that do not interfere with the ligands detectable by the assay. Some of these may be located on proteins other than the reaction center proteins. Nevertheless, we feel His 337 on the carboxyl end of D1 (Zurawski et al., 1982; Trebst, 1986) is still a viable candidate for the carboxypeptidase A sensitive site. We should note here again that while the assignment of the four high-affinity Mn-binding sites observed here to sites involved in photoligation of Mn during photoactivation or to functional Mn sites is attractive, it will require a battery of biochemical measurements perhaps on site-specific mutants to confirm or deny this point. Nevertheless, the fact that removal of two of the four functional Mn uncovers two (the EDC-sensitive ones) of the four high-affinity sites (Preston & Seibert, 1991) is suggestive of a very close relationship. For this reason we feel that the identification of His 337 on D1 as a possible Mn-binding site is notable.

In these experiments we have characterized four independent high-affinity Mn-binding sites (ligands) in PS II when high concentrations of DPC are present. Two of the sites can be degraded specifically, a third site can be degraded in conjunction with one of the first two sites, but the fourth site as yet cannot be degraded with proteases. Finally, we have identified one amino acid, His 337 on the D1 protein, as a strong candidate for one of the high-affinity Mn-binding sites and perhaps as the source of one ligand for binding functional Mn.

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