Use of a Novel Histidyl Modifier To Probe for Residues on Tris-Treated Photosystem II Membrane Fragments That May Bind Functional Manganese†

Maria L. Ghirardi,‡ Christopher Preston,§ and Michael Seibert*,‡

Basic Sciences Center, National Renewable Energy Laboratory, Golden, Colorado 80401, and Department of Plant Protection, Waite Agricultural Research Institute, The University of Adelaide, Glenn Osmond, S.A. 5064, Australia

Received February 13, 1998; Revised Manuscript Received July 29, 1998

ABSTRACT: In this paper, we investigate the effects of histidyl amino acid modification on high-affinity Mn binding to photosystem II (PSII) using methods similar to those used in the preceding paper [Ghirardi et al. (1998) *Biochemistry 37,* 0000] for carboxyl amino acid modification. Given the rather low specificity of diethyl pyrocarbonate (DEPC) for histidine modification, we modified Tris-washed PSII membranes with a novel and more specific histidyl modifier, platinum(II) (2,2′:6′,2″-terpyridine) chloride (Pt-TP). Both the "diphenylcarbazide (DPC)-inhibition assay" and single-turnover flash approaches were used. The concentration dependence of Pt-TP modification on steady-state measurements shows two types of interactions, each accounting for about half of the full effect. At concentrations <50 μM, Pt-TP modifies mostly histidyls and abolishes half of the observed Mn inhibition of DPC-mediated 2,6-dichlorophenolindophenol (DCIP) photoreduction (equivalent to two high-affinity, Mn-binding ligands). This effect can be blocked by addition of Mn^{2+} during Pt-TP modification. Double-modification experiments with DEPC and Pt-TP demonstrate that both modifiers affect the same observable histidyl residues in PSII. Above 50 μM, Pt-TP modifies mostly cysteines (or histidines in a more hydrophobic environment) and has an additional effect on the reducing side of PSII that (a) does not involve Mn binding and (b) results in the *apparent* abolishment of all four of the Mn-binding ligands detected by the DPC-inhibition assay. Single-flash experiments show that histidyl modification does not eliminate the binding of the high-affinity, photooxidizable Mn^{2+} to Asp170 on D1 (nor does it significantly affect high-affinity DPC photooxidation), but it does decrease the binding affinity (K_d) of that Mn from 0.6 to 1.5 μM, particularly at lower (<50 μM Pt-TP) concentrations. Double-modification experiments also demonstrate that the lower affinity, photooxidizable Mn-binding site, uncovered when the high-affinity site is modified with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) [see Ghirardi et al. (1998)], is not associated with a histidyl ligand. Three nonphotooxidizable, high-affinity Mn^{2+} ions bind to a second carboxyl and two histidyl ligands, and these Mn are not photooxidized by a flash even when the ligand to the photooxidizable Mn is modified by EDC. Proteolytic enzyme studies indicate that the two histidyl ligands identified by the DPC-inhibition assay are probably His337 on D1 and His 339 on D2, but His 332 on D1 is not eliminated.

Covalent modification of amino acid side chains has been used commonly to investigate the function of specific residues in a variety of cellular and membrane proteins (*1, 2*). The involvement of histidine residues in binding Mn on photosystem II (PSII)[1] proteins was originally investigated by chemically modifying histidyl ligands with diethyl pyrocarbonate (DEPC). Histidines were shown to be essential for photoactivation of O_2 evolution (*3*), as well as for binding at least two high-affinity Mn^{2+} ions when the tetrameric Mn cluster required for water-splitting function was removed (*4*−*8*). Taken together, these studies suggested that one or more histidine residues on the lumenal side of the PSII membrane provided ligands required for assembling or binding the functional Mn cluster. Unfortunately, DEPC is not very specific for histidine, and, depending on the assay conditions, it may also modify cysteines, tyrosines, and lysines (*9*). To address this issue, a new histidyl modifier, platinum(II) (2,2′:6′,2″-terpyridine) chloride, hereafter referred to as Pt-TP, was developed recently (*10, 11*) as an alternative to DEPC. This compound exhibits marked selectivity toward only histidines and cysteines and is completely unreactive with other amino acid residues including methionine, serine, glutamate, and aspartate. An additional advantage of Pt-TP is its noninva-

† This work was sponsored by the Division of Energy Biosciences, Office of Basic Energy Sciences, U.S. Department of Energy (M.S.).

‡ National Renewable Energy Laboratory.

§ The University of Adelaide.

[1] Abbreviations: Cyt b_{559}, cytochrome b_{559}; D1 and D2, major reaction center proteins of photosystem II; DCIP, 2,6-dichlorophenolindophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; F_{final}, fluorescence yield detected after decay of the flash-induced F_{max}; F_{max}, maximum fluorescence yield following actinic flash excitation; K_d, dissociation constant of a substrate−enzyme complex; F_V, variable fluorescence yield; K_M, Michaelis−Menten constant for an enzymatic reaction; MES, 2-(*N*-morpholino)ethanesulfonic acid; PS, photosystem; Pt-TP, platinum(II) (2,2′:6′,2″-terpyridine) chloride [others have used the abbreviation $[Pt(trpy)Cl]^+$ for this compound]; Tris, tris(hydroxymethyl)aminomethane; V_{max}, maximum initial rate of an enzymatic reaction; Y_Z, tyrosine 161 on the D1 protein.

S0006-2960(98)00359-6 CCC: $15.00
Published on Web 09/11/1998

sive character since it reacts only with amino acid side chains that are exposed to the surrounding medium (*11*). Furthermore, chemical modification of histidines and cysteines occurs at much lower Pt-TP than DEPC concentration, the extent of modification can be monitored easily by UV spectroscopy, and the nature of the ligand modified and its environment can be deduced by measuring the relative amplitudes of different absorption bands (*11*).

Our previous work has characterized the high-affinity, Mn-binding site in Tris-washed membranes (lacking the tetrameric Mn cluster and the extrinsic proteins) using an assay based on the ability of exogenous Mn to noncompetitively inhibit diphenylcarbazide (DPC) photooxidation by PSII (*4, 5, 7, 8*). DPC is not photooxidized by PSII preparations containing an intact Mn cluster, and the high-affinity Mn site is uncovered with the removal of the cluster (*12*). The DPC-inhibition assay measures the effect of added micromolar concentrations of $MnCl_2$ on the initial rate of DCIP photoreduction measured in the presence of 200 mM DPC, which normally acts as a donor to $Y_Z^{\bullet}$. As mentioned in the accompanying paper (*13*), the high-affinity Mn site detected by the DPC-inhibition assay is composed of four components (or ligands) associated with two carboxyl and two histidyl amino acid residues each of which binds an independent Mn ion (*6*−*8*). If all four ligands to Mn that we have previously characterized are present, $MnCl_2$ addition results in a decrease of about 50% in the rate of DCIP photoreduction under our experimental conditions. However, if one or more of the ligands are missing due to chemical modification (or protease treatment), then addition of $MnCl_2$ will result in a smaller decrease in the rate of DCIP photoreduction (e.g., see Figure 2, ref *13*). Only one of the four bound Mn undergoes photooxidation under the assay conditions (*14*), and this photooxidizable Mn is bound by a carboxyl residue, probably Asp170 on the D1 protein, as concluded in the accompanying paper (*13*). Modification of that carboxyl residue uncovers a new lower affinity, Mn-binding site that could be a histidine (*13*). The other three ligands bind Mn that is not photooxidized under the assay conditions, and some of these Mn may be competitive with Zn and Co (*14*).

In this paper, we demonstrate that Pt-TP at low concentrations, like DEPC, modifies two of the Mn ligands on the oxidizing side of PSII as detected using the DPC-inhibition assay. These ligands are identified as histidines, but flash studies show that the modifier does affect the affinity of the photooxidizable Mn. Double-modification experiments show that the lower affinity, Mn-binding site uncovered by EDC treatment (*13*) is not associatiated with a histidyl ligand. On the other hand, modification with Pt-TP at high concentration has a secondary effect on the reducing side of PSII, due mainly to modification of cysteine residues (or histidyls in a hydrophobic environment) that do not interfere with the binding of Mn. Proposed identification of the histidine ligands will be discussed.

MATERIALS AND METHODS

Thylakoids and PSII membrane fragments were prepared from market spinach as before (*14*) and resuspended in K4 buffer (20 mM MES, pH 6.5, containing 400 mM sucrose, 15 mM NaCl, and 5 mM $MgCl_2$). *Scenedesmus obliquus* LF-1 cells were grown and PSII membranes prepared as described by Metz and Seibert (*15*). Tris-washing of PSII membranes and determination of chlorophyll concentration were described by Ghirardi et al. (*14*). Treatment of membranes with the histidyl modifier DEPC and with the carboxyl modifier EDC was done as previously reported (*7, 8*). Modification of the membranes with the new histidyl modifier, Pt-TP, was done in buffer A (20 mM MES buffer, pH 6.5, containing 400 mM sucrose and 20 mM NaCl). Tris-washed PSII membranes were washed once in buffer A, resuspended to a final concentration of 120 μg of Chl/mL, and incubated with different concentrations of Pt-TP for 1 h at room temperature in the dark. The reaction was terminated by the addition of 2 volumes of ice-cold buffer A supplemented with 10 mM D,L-histidine. The membranes were collected by centrifugation (40000*g*, 10 min), washed once, and either resuspended in buffer A or stored in K4 buffer. When $MnCl_2$ was added to the Pt-TP modification buffer, a second Tris-washing was performed after the chemical modification to remove any Mn that had become associated with the membrane. DEPC was purchased from Sigma; Pt-TP as the chloride salt was obtained from Aldrich (catalog no. 28,809-8).

Carboxypeptidase treatment of PSII membranes was performed with either or both carboxypeptidase A and carboxypeptidase B. Tris-washed spinach PSII membranes at 200 μg of Chl/mL were incubated with 1:1 w/w carboxypeptidase A/Chl and/or 1:4 w/w carboxypeptidase B/Chl for 1 h in buffer A at 20 °C in the dark. Phenylmethanesulfonyl fluoride at 1 mM was added to the incubation buffer to inhibit possible endopeptidase activity. The reaction was stopped by addition of 20 volumes of ice-cold buffer B containing 4 mM 1,10-phenanthroline. The membranes were collected by centrifugation (40000*g*, 10 min), washed once in buffer A, and either resuspended in buffer A or stored in K4 buffer.

Absorption spectra of Pt-TP-modified samples were taken with a Carey 5E UV−Vis−NIR spectrophotometer (Varian, Australia). Initial rates of DCIP photoreduction were calculated from measurements of the initial slope of the kinetics at 600 nm within 3 s of the commencement of steady-state illumination using the split-beam mode of a DW2a spectrophotometer as described (*7, 8*). Flash-probe fluorescence measurements were done with a home-built instrument as described before (*13, 14*).

RESULTS

Brothers and Kostic (*11*) used Pt-TP to modify amino acid residues on cytochrome *c* from both *Candida krusei* and bakers' yeast. This work established the suitability of the chemical modifier for binding only to surface-exposed residues without affecting the basic structure and function of the protein. Based on this work, we examined the effect of Pt-TP modification on the high-affinity, Mn-binding site detectable by the DPC-inhibition assay. Table 1 shows that modification of Tris-washed PSII membranes with 50 μM Pt-TP caused a decrease in the DCIP photoreduction rate by 200 μM DPC to 60% of the rate measured in unmodified membranes. This decrease is comparable to that observed upon treatment of the same membranes with 10 mM EDC

Table 1: Initial and Relative Rates of DCIP Photoreduction by Control Tris-Washed PSII Membranes That Were Either Unmodified or Modified with 50 μM Pt-TP[a]

	rate of DCIP photoreduction [μmol of DCIP (mg of Chl)$^{-1}$ h^{-1}]		
electron donor	control PSII	Pt-TP-modified PSII	modified/ unmodified
200 mM DPC	196	116	0.59
10 mM $MnCl_2$	33	29	0.88

[a] The donor was either 200 μM DPC or 10 μM $MnCl_2$.

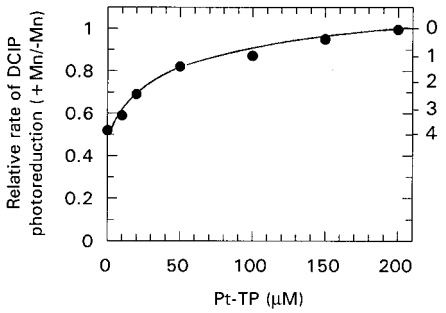

FIGURE 1: Relative initial rates of DCIP photoreduction by 200 μM DPC measured in the presence and absence of added 10 μM $MnCl_2$. The measurements were done with Tris-washed spinach PSII membrane fragments either unmodified or modified with different concentrations of the histidine chemical modifier Pt-TP. Two rise components are apparent. The right *y*-axis indicates the apparent number of unmodified components (Mn-binding ligands) associated with the high-affinity Mn-binding site that correspond to the given relative rates at each Pt-TP concentration, according to Preston and Seibert (*7*, *8*).

(*13*). On the other hand, as expected, the rate of DCIP photoreduction by 10 μM $MnCl_2$ was not significantly affected by the same Pt-TP treatment since only the Mn bound to a carboxyl residue is photooxidized under the assay conditions (*13*). Since DPC donation does not depend on the integrity of the high-affinity, Mn^{2+}-binding site (DPC and Mn^{2+} interact in a noncompetitive manner; *7*), chemical modification of Mn ligands should not significantly inhibit DPC donation to DCIP (*7*, *8*, *14*, *16*).

Figure 1 shows the relative rate of DCIP photoreduction by DPC in the presence and absence of 10 μM $MnCl_2$ as a function of Pt-TP concentration present during modification of Tris-washed membranes. The *y*-axis on the right side of the figure indicates the apparent number of unmodified Mn ligands detectable by the DPC-inhibition assay. Treatment of membranes with increasing concentrations of Pt-TP results in a decreasing inhibitory effect of Mn^{2+} on DCIP photoreduction, due to an apparent progressive loss of Mn-binding ligands. On first inspection, these data do not appear to support our previous observation that modification of histidine residues removes only two of four Mn ligands detectable by the DPC assay (*8*) since four ligands seem to be eliminated at high Pt-TP concentration. However, further analysis of Figure 1 suggests that Pt-TP modification of PSII membranes involves two different sites of action since there seems to be a distinct change in slope at about 40−50 μM Pt-TP. We will show that low concentrations of Pt-TP ($\leq$50 μM) modify Mn-binding residues, but high concentrations ($\geq$50 μM) modify residues that do not bind Mn.

To demonstrate this, we initially analyzed the UV absorption difference spectra of Pt-TP-modified minus unmodified membranes to determine whether histidines or cysteines were being preferentially modified in each concentration range. Figure 2 shows the difference spectra of PSII membranes modified with increasing concentrations of Pt-TP. The spectra show absorption maxima at 247, 280, 328, and 342 nm which closely match the four wavelengths described for modified histidyls in model compounds (*10*). Covalent binding of Pt-TP to histidyl residues in model compounds is characterized by a $\Delta A_{342}/\Delta A_{328}$ ratio of 1.32, while Pt-TP bound to cysteines yields a ratio on the order of 1.14 (*10*, *11*). However, a $\Delta A_{342}/\Delta A_{328}$ ratio of about 1.1 could also indicate modification of histidyl residues in a more hydrophobic environment than those characterized by a ratio of 1.32 (*11*). Figure 3 shows the effect of Pt-TP concentration during treatment on the $\Delta A_{342}/\Delta A_{328}$ ratio in spinach PSII membranes. When the treatment is done in 20 μM Pt-TP or less, the ratio was over 1.30 (note that the 328 nm peak is in the curve noise and not well-resolved at these low Pt-TP concentrations and thus the ratios calculated represent *minimum* values), indicating that mostly histidyls were being

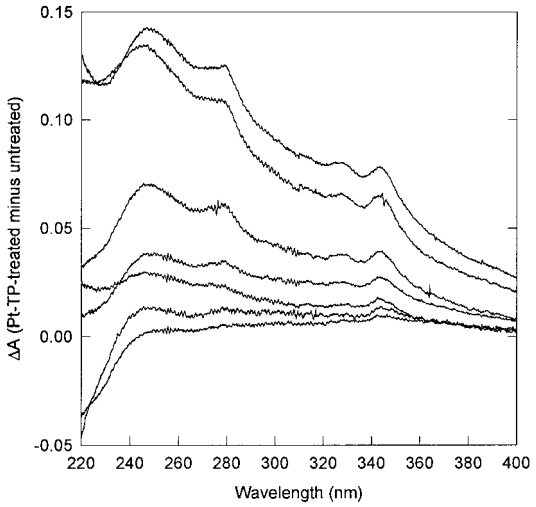

FIGURE 2: Absorption difference spectra of Tris-washed spinach PSII membranes modified with increasing concentrations of Pt-TP minus unmodified membranes. The absorption spectra were first normalized at the maximum chlorophyll absorbance peak (about 680 nm) and then subtracted from the spectrum corresponding to the unmodified sample. The individual difference spectra represent membranes modified with, respectively, from the bottom, 10, 20, 30, 50, 100, 150, and 200 μM Pt-TP. Major absorption difference peaks can be observed at 247, 280, 328, and 342 nm, close to those described by Ratilla et al. (*10*) for model compounds.

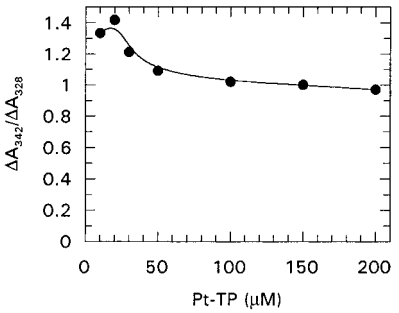

FIGURE 3: Ratio between the absorbance changes (modified minus unmodified) at 342 and 328 nm determined with PSII membranes as a function of the Pt-TP concentration used during the treatment. A ratio of 1.32 is characteristic of modified histidines, and a ratio of 1.14 represents modified cysteines or modified histidines in a more hydrophobic environment (*10*, *11*).

Table 2: Ratio of the Rate of DCIP Photoreduction by 200 μM DPC Measured in the Presence and Absence of 10 μM $MnCl_2$, and the Corresponding Apparent Number of Mn-Binding Components (Ligands), as per Preston and Seibert (*7*, *8*)[a]

Tris-washed PSII membranes	+Mn/−Mn	remaining Mn-binding ligands
spinach control	0.50	4
treated with 10 mM EDC	0.75	2
treated with 10 mM EDC + 5 mM $MnCl_2$	0.50	4
treated with 200 μM Pt-TP	0.98	0
treated with 200 μM Pt-TP + 5 mM $MnCl_2$	0.74	2
LF1 mutant control	0.72	2
treated with 10 mM EDC	0.99	0
treated with 10 mM EDC + 5 mM $MnCl_2$	0.75	2
treated with 200 μM Pt-TP	0.99	0
treated with 200 μM Pt-TP + 5 mM $MnCl_2$	0.98	0

[a] Samples consisted of Tris-washed PSII membranes either from spinach or from the *S. obliquus* LF1 mutant (see Materials and Methods).

Table 3: Ratio of the Relative Rate of DCIP Photoreduction by 200 μM DPC Measured in the Presence and Absence of 10 μM $MnCl_2$, and the Corresponding Number of High-Affinity, Mn-Binding Ligands[a]

thylakoid treatment	+Mn/−Mn	remaining Mn-binding ligands
control	0.52	4
treated with 500 μM DEPC	0.52	4
treated with 300 μM Pt-TP	0.76	2
treated with 20 μM Pt-TP	0.54	4
treated with 300 μM Pt-TP, fractionated into PSII membranes, Tris-washed, then treated with 500 μM DEPC	0.98	0

[a] Thylakoid membranes in this experiment were either untreated (control) or treated with histidyl modifiers, and the thylakoids were then fractionated into PSII membranes for assay. The last row describes Tris-washed PSII membranes derived from Pt-TP-treated thylakoids that were subjected to a second modification with 500 μM DEPC prior to assay.

modified. However, when the concentration of Pt-TP was increased above 50 μM, the ratio dropped to 1.1 or lower, suggesting that the modification of cysteines (or histidyls in a hydrophobic environment) was becoming more prevalent. Given that cysteines are not known to bind Mn and that histidines in Tris-washed membranes are exposed to the hydrophilic aqueous medium, these results suggest that Mn-binding ligands must be modified by the lower concentrations of Pt-TP only.

To further investigate the concentration-dependent effect of Pt-TP modification on Mn ligands, we examined whether addition of $MnCl_2$ to the modification buffer protected all apparent ligands from Pt-TP modification. Both spinach PSII membranes (which contain the full complement of four Mn ligands, two carboxyls, and two histidyls) and PSII-enriched membranes isolated from the *Scenedesmus obliquus* LF1 mutant were used in this experiment. This mutant has only two exposed Mn ligands that are detectable by the DPC-inhibition assay, and these have been determined to be carboxyl residues (*4*, *7*, *8*). Table 2 shows the relative rate of DCIP photoreduction by DPC measured in the presence and absence of $MnCl_2$ (+Mn/−Mn) using control Tris-washed PSII membranes, membranes modified with 10 mM EDC in the presence or absence of 5 mM $MnCl_2$, and membranes modified with 200 μM Pt-TP in the presence or absence of 5 mM $MnCl_2$. Membranes modified in the presence of $MnCl_2$ were subsequently Tris-washed to expose the protected Mn ligands prior to analysis using the DPC-inhibition assay. The last column lists the corresponding number of unmodified Mn-binding ligands detected by the DPC-inhibition assay. The full complement of available Mn ligands is detected by the assay in control Tris-washed PSII membranes (four ligands in spinach and two in the LF1 mutant). Modification of the control membranes with 10 mM EDC reduces the number of detectable Mn ligands to two in spinach and to zero in the LF1 mutant due to the specificity of the modifier for carboxyl residues (*7*). However, when the EDC modification is done in the presence of $MnCl_2$, both carboxyl ligands are protected and subsequently can be detected in spinach or LF-1 membranes washed with Tris for the second time. On the other hand, modification of the membranes with 200 μM Pt-TP in the absence of $MnCl_2$ eliminates all apparent Mn ligands, as in Figure 1. The results are qualitatively the same in spinach and in the LF1 mutant, even though spinach membranes have only two histidyl ligands and mutant membranes lack histidyl ligands to high-affinity Mn. Since the addition of $MnCl_2$ to the modification buffer protects only half of the ligands in spinach membranes, and none in the LF1 mutant membranes, these results clearly demonstrate that the modification effects of high concentrations ($\geq$50 μM) of Pt-TP on the DPC-inhibition assay are not related to the binding of Mn.

To further define the effect of high concentrations of Pt-TP on the DPC-inhibition assay, we treated thylakoid membranes with two types of histidyl modifiers, Pt-TP and/or DEPC. The thylakoids were subsequently fractionated into PSII membranes and then Tris-washed prior to the application of the DPC-inhibition assay. In contrast to PSII preparations, in which both the oxidizing and reducing sides of the PSII membrane are exposed to the medium (*17*), the oxidizing side of PSII in thylakoid membranes is sequestered in the lumen, and cannot be influenced by modifiers that only affect surface-exposed residues. Table 3 shows that four Mn-binding ligands are detected in Tris-washed PSII membranes derived from unmodified thylakoids, as expected. If thylakoids are treated with 500 μM DEPC prior to fractionation and Tris-washing, the four Mn-binding ligands are still intact, indicating that DEPC does not modify any residues, detectable by the assay, on the reducing (stromal) side of PSII. However, thylakoids modified with 300 (but not 20) μM Pt-TP, prior to fractionation and Tris-washing, show that only two apparent Mn-binding ligands remain. This suggests that, in contrast to DEPC, high concentrations of Pt-TP must affect residues on the stromal side of PSII. This hypothesis was confirmed by subjecting Tris-washed PSII membranes derived from Pt-TP-modified thylakoids, as above, to a second modification with 500 μM DEPC. This second treatment should further modify histidyl, Mn-binding residues located on the oxidizing (lumenal) side of PSII (*8*) that were not affected by the previous treatment with Pt-TP. As expected, no Mn-binding ligands were detected under these conditions, indicating that high concentrations of Pt-TP do indeed affect residues on the stromal side of PSII, probably cysteines (see Figure 3), which do not bind Mn but whose modification affects the apparent number of Mn-

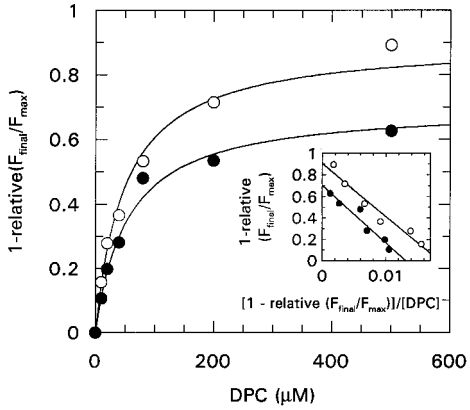

FIGURE 4: The [1 − relative (F_{final}/F_{max})] ratio as a function of added DPC using unmodified control (open circles) and 50 μM Pt-TP-modified (closed circles), Tris-washed PSII membranes. [1 − relative (F_{final}/F_{max})] is a measure of the number of PSII centers undergoing an initial light-induced charge separation reaction that do not recombine. Inset: Scatchard plot of the data. The *y*-intercept is an estimate of the number of PSII centers undergoing a charge recombination reaction following an actinic flash; the slope of the line is an estimate of the affinity (K_d) of DPC for the PSII membranes.

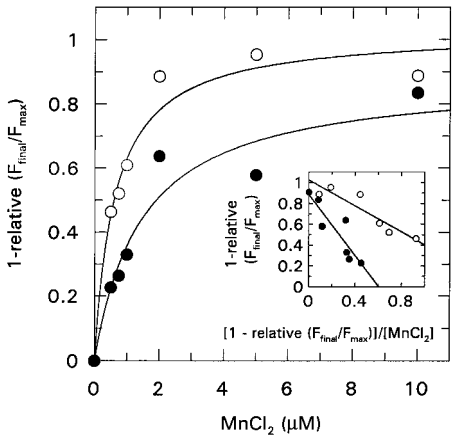

FIGURE 5: The [1 − relative (F_{final}/F_{max})] ratio as a function of added $MnCl_2$ using unmodified control (open circles) and 50 μM Pt-TP-modified (closed circles), Tris-washed PSII membranes. [1 − relative (F_{final}/F_{max})] is a measure of the number of PSII centers undergoing an initial light-induced charge separation reaction that do not recombine. Inset: Scatchard plot of the data. The *y*-intercept is an estimate of the number of PSII centers undergoing a charge recombination reaction following an actinic flash; the slope of the line is an estimate of the binding affinity (K_d) of Mn for key amino acid residues on the PSII membranes.

binding sites detected by the DPC-inhibition assay. *The reader is cautioned about using Pt-TP at concentrations higher than 50 μM if high-affinity Mn ligands are to be examined.*

The accompanying paper (*13*) shows that modification of carboxyl residues with EDC has a selective effect on Mn binding and photooxidation. We demonstrated the point clearly by titrating the final fluorescence yield following a short saturating flash using either DPC or $MnCl_2$ as a donor. Similar experiments are repeated here except with low-concentration Pt-TP modification in order to probe for the effects of histidyl residues on both DPC and Mn binding and photooxidation potential. Figure 4 shows the effect of increasing concentrations of DPC on [1 − relative (F_{final}/F_{max})] (see the accompanying paper for details about this flash-probe fluorescence technique) in unmodified, Tris-washed PSII membranes and in Tris-washed membranes modified with 50 μM Pt-TP. The inset is a Scatchard plot of the data in Figure 4. The slopes of the lines fitted to the data points in the inset are an estimate of the binding affinity of DPC to the membranes, and the *y*-intercept is a measure of the relative number of active centers. We obtained a value of 49 μM for the binding affinity (K_d) of DPC to untreated membranes and 54 μM DPC for Pt-TP-treated membranes. These values demonstrate that Pt-TP treatment does not significantly change the affinity of DPC for the membranes. On the other hand, the relative number of centers that photooxidize DPC decreased from about 90% to 70% (see the respective points where the curves cross the ordinate in the inset) following Pt-TP treatment, indicating a secondary effect of the modifier, as suggested by Table 1.

Figure 5 shows that, in contrast to the results with EDC modification (see the accompanying paper), Pt-TP modification does not inhibit $MnCl_2$ photooxidation to a significant extent. The inset does show, however, that the treatment causes an increase in the K_d for photooxidizable Mn (from 0.62 to 1.47 μM), as seen by an increase in the slope of the line fitted to the Pt-TP-treated membranes. This increase in K_d had little influence on the number of centers observable

Table 4: Pt-TP Titration of the Estimated Binding Affinity (K_d) for the High-Affinity Photooxidizable Mn As Determined from Flash-Induced Fluorescence Measurements in Tris-Washed PSII Membranes from Spinach

[Pt-TP] (μM)	K_d, high affinity (μM)	[Pt-TP] (μM)	K_d, high affinity (μM)
0	0.62	50	1.47
10	0.75	100	1.48
20	1.05	200	1.90

after the modification. We next studied the effect of different concentrations of Pt-TP on the binding of photooxidizable Mn in Table 4 and found that the K_d is much more sensitive to Pt-TP modification at concentrations below 50 μM than above 50 μM. All of these results confirm our hypothesis that only Mn bound to a carboxyl residue is photooxidizable (*13*). However, it is clear that modification of histidyl residues near the carboxyl that binds the photooxidizable Mn affects the K_d of that Mn. The smaller effect of higher Pt-TP concentrations on the K_d of Mn also points to a secondary action of Pt-TP that does not involve Mn binding as indicated above.

In the accompanying paper, we were not able to identify the lower affinity ligand to Mn uncovered when the carboxyl ligand (Asp170 on D1) to the photooxidizable Mn was modified. To test whether this lower affinity ligand is a histidine, we did a double-chemical-modification experiment. Tris-washed PSII membranes were initially treated either with the carboxyl modifier EDC or with the histidyl modifier Pt-TP, and flash-induced fluorescence measurements were performed to estimate the K_ds for photooxidizable Mn after each treatment. Each sample was then complementarily treated either with Pt-TP or with EDC in order to completely eliminate high-affinity, Mn-binding sites detected by the DPC-inhibition assay (*7*, *8*) including the high-affinity photooxidizable Mn bound by a carboxyl residue. These doubly modified samples were used for a second round of flash-induced fluorescence measurements to determine whether

Table 5: Maximum Fluorescence Yield (F_{max}) and Binding Affinity (K_d) for DPC and Mn As Determined by Flash-Probe Fluorescence Measurements[a]

PSII membrane treatment	200 μM DPC		10 μM $MnCl_2$	
	F_{max}	K_d (μM)	F_{max}	K_d (μM)
control	0.780	78.4	0.816	1.0
control, buffer treated once (1st modification control)	0.761	118.4	0.775	0.8
50 mM EDC modified	0.663	80.8	0.453	20.4
50 μM Pt-TP modified	0.636	100.0	0.708	3.6
control, buffer treated twice (2nd modification control)	0.733	77.6	0.764	0.8
50 mM EDC + 50 μM Pt-TP treated	0.542	54.4	0.415	13.7
50 μM Pt-TP + 50 mM EDC treated	0.548	124.0	0.445	38.0

[a] Tris-washed PSII membranes were either unmodified (control) or modified with EDC, Pt-TP, or both.

Table 6: DPC-Inhibition Assay Performed on Tris-Washed Spinach PSII Membranes after Treatment with Carboxypeptidase (CPase) A, a Mixture of CPase A and B, the Carboxyl Modifier EDC, and the Histidyl Modifier DEPC[a]

Tris-washed PSII membranes	+Mn/−Mn	remaining Mn-binding ligands
control	0.52	4
treated with CPase A	0.62	3
treated with CPase A and CPase B	0.73	2
treated with CPase A and CPase B, then treated with 10 mM EDC	1.01	0
treated with CPase A and CPase B, then treated with 500 μM DEPC	0.76	2

[a] Note that two Mn ligands are removed when both peptidases are present and the lost ligands are histidyls.

the modification of both carboxyl and histidyl residues inactivated all ligands to photooxidizable Mn.

Table 5 summarizes these results. Modification of Tris-washed PSII membranes with either 50 mM EDC or 50 μM Pt-TP eliminates 15−18% of the centers that photooxidize DPC, compared to the loss of only 2.5% of the centers estimated from control samples submitted to the same conditions (minus the modifiers). A second chemical modification with either Pt-TP or EDC increases the number of inactivated DPC-photooxidizing centers to about 30%, compared to 6% for buffer-treated control samples. The affinity of the membranes for DPC varies between 54 and 124 μM in modified as well as unmodified samples. This variation does not seem to depend on the type of treatment, but may instead reflect other experimental conditions. These results indicate that neither EDC nor Pt-TP modification has a major effect on DPC photooxidation in these membranes; thus, this activity does not depend on either carboxyl (*13*, *16*) or histidyl residues. The effect of the same chemical modifications on high-affinity Mn binding, however, is very different (Table 5). EDC modification, either before or after Pt-TP, eliminates about 50% of the high-affinity, Mn-binding centers and uncovers the lower affinity, Mn-binding component with K_d of 14−40 μM, consistent with the results of Table 2 in the accompanying paper (*13*). Modification of the same membranes with Pt-TP, either before or after EDC, results in inactivation of only 5−8% of the Mn-binding centers, and in a small increase in the K_d of the remaining centers. These results confirm that the high-affinity photooxidizable Mn is bound by a carboxyl residue, but rule out the possibility that the lower affinity photooxidizable Mn ligand detected after EDC modification of the membranes is a histidyl residue.

Based on their sensitivity to different proteases, the two histidyl ligands to high-affinity Mn were identified as His337 on D1 and possibly His339 on D2 (*8*). To more accurately establish their identity, we submitted Tris-treated PSII membranes to digestion by carboxypepidase A, carboxypepidase B, or a combination of both. Carboxypeptidase A alone degrades a quarter of the observable high-affinity Mn site (*8*) while carboxypeptidase B alone has no effect (data not shown). Following treatment with a mixture of both enzymes, however, Mn inhibition of DPC-supported DCIP photoreduction was reduced from 50% to about 25% (Table 6), indicating the loss of two ligands. Subsequent treatment of this material with DEPC had no further effect whereas subsequent treatment with EDC completely eliminated Mn inhibition of DPC-supported DCIP photoreduction. Therefore, the two proteases in combination remove half the observable high-affinity Mn ligands from the membranes (Table 6), and those ligands are histidines. Their possible identity is discussed later.

DISCUSSION

Histidine residues have been implicated as important structural components associated with Mn binding near the catalytic site of the O_2-evolving complex of PSII from chemical modification, proteolytic enzyme treatment, and classical mutant studies (*3*−*8*). Electron spin−echo envelope modulation (ESEEM; *18*) and electron−nuclear double resonance (ENDOR; *19*) studies have confirmed the earlier work by suggesting that one or more nitrogen atoms provide ligands to the Mn cluster itself. Tang et al. (*20*), using ESEEM, further showed that these nitrogen ligands are most likely histidine imidazole nitrogens. Site-directed mutants have further supported this evidence (*21*−*25*). His337 on D1 and His339 on D2 were identified as potential Mn ligands from the biochemical studies and studies of the *Scenedesmus obliquus* LF1 mutant (*7*, *8*), while His332 and His337 on D1 were suggested as a result of site-directed mutant studies (*21*−*25*). A histidine residue, possibly His190 on D1 (*26*), can be photooxidized in the absence of functional Mn (*27*, *28*), in Ca^{2+}-depleted PSII membranes (*29*, *30*), and in PSII O_2-evolving core complexes (*20*). It has also been suggested that this residue binds Mn (*31*). However, more recent work (*32*) disputes these results and attributes the photooxidizable residue to Y_Z instead.

Our current study has shown that Pt-TP, a histidyl modifier, has two effects on PSII depending on the concentration of the modifier during membrane treatment. At low concentrations ($\leq$50 μM) of the modifier, Pt-TP affects the lumenal side of the PSII membrane and modifies two histidyl residues that bind Mn with high affinity (Figures 1−3; Table 3). Modification under these conditions is prevented by the presence of $MnCl_2$ in the treatment buffer (Table 2), indicating specificity for Mn. Furthermore, Pt-TP treatment does not prevent the photooxidation of DPC (Figure 4) nor the binding of photooxidizable Mn (Figure 5; Table 4) to PSII membranes, but does slightly decrease the affinity of the latter. Treatment with Pt-TP also fails to remove the lower affinity photooxidizable Mn detected after EDC

treatment of Tris-washed PSII membranes (*13*), demonstrating that the residue that binds that particular Mn is not a histidine. This in turn leads us to question the conclusion of Blubaugh and Cheniae (*33*) and Magnuson and Andreasson (*34*) that the high-affinity component of the Mn titration of the DCIP photoreduction curve in EDC-treated samples is due to photooxidizable Mn bound to histidyl residues. It is important to point out that the residual photooxidizable Mn that donates to $Y_Z^{\bullet}$ after chemical modification with EDC is distinct from the normal low-affinity, Mn-donation site to PSII (*14*). We conclude that treatment with low concentrations of Pt-TP results in the modification of histidine residues that contribute ligands to the high-affinity binding of nonphotooxidizable Mn to PSII in a manner similar to that of DEPC.

High concentrations of Pt-TP ($>50\ \mu$M) have an additional effect that is localized on the stromal side of the membrane (Table 3). This low-affinity modification site detected by the DPC-inhibition assay was not protected by $MnCl_2$ (Table 2) and is apparently associated with an ancillary phenomenon involving the modification of a cysteine residue(s) (Figures 2 and 3), the only other residue that can be modified by Pt-TP, or histidines in a hydrophobic environment (*11*). We emphasize that Pt-TP modification at high concentration does not affect additional residues involved in Mn binding. Modification of this (these) cysteine residue(s) may facilitate the reaction between bound Pt-TP and an electron-transport component, thus affecting electron transport, or it may cause structural changes due to the introduction of too many Pt-TP radicals to the protein structure of the PSII complex, influencing its function. This is supported by the decrease in the rate of of both Mn- and DPC-supported DCIP photoreduction observed upon modification of the membranes with high concentrations of Pt-TP (52 and 44% of the untreated rate with Mn and DPC, respectively, in samples treated with 200 μM Pt-TP; data not shown). This decrease in electron-transport rate was not apparent if silicomolybdate was used as an electron acceptor (data not shown), which suggests that inhibition is on the reducing side of PSII beyond Q_A (*35*).

The full complement of high-affinity, Mn-binding sites [four components detectable by the DPC-inhibition assay associated with four ligands, including two histidyls, that can bind Mn (*7*, *8*)] can be observed in the isolated D1−D2−Cyt b_{559} reaction center complex (*4*). This preparation consists of five proteins, only two of which, D1 and D2, contain histidine residues near the carboxyl end protruding from the lumenal side of the membrane (*36*−*41*). From the folding model of D1 and D2 proposed by Trebst (*37*), we believe that only three histidine residues are primary candidates for ligands to high-affinity Mn detectable by the DPC-inhibition assay. These are His332 and His337 on D1, and His339 on D2. Based on the observation that trypsin, V8 protease, and carboxypeptidase A treatments each removed the same DEPC-sensitive histidyl ligand to Mn, we concluded that this ligand is located on the carboxyl end of D1 beyond Arg 334 (carboxypeptidase A cannot hydrolyze Arg residues, and His339 on D2 is protected by Arg351) and is His337 (*8*). The other ligand may be His339 on D2, based on symmetry arguments and the location of a subtilisin cleaving site a few residues to the amino side of this histidine (*8*). Nixon et al. (*42*), using single-turnover flash studies of site-specific mutants, have suggested that His332 on D1 is a good candidate for a ligand to a functional Mn. This assignment has been supported by other studies using site-specific mutants (*22*, *24*, *43*), and at the very least His332 on D1 seems to be required for the stable assembly and function of the Mn center (*42*). Recently, Chu et al. (*25*) also identified His337 on D1 as a potential ligand to Mn.

In this paper, we have extended our studies by treating spinach PSII membranes with a combination of carboxypeptidase A and carboxypeptidase B (the latter hydrolyzes arginines) in Table 6. These two proteases complement each other and will sequentially remove amino acids from the carboxyl ends of proteins until blocked by the tertiary structure of the protein (*44*). Our carboxypeptidase A and B experiments, however, cannot differentiate between His332 on D1 or His339 on D2 as the second high-affinity ligand detected by the DPC-inhibition assay, but they do eliminate all other histidines on reaction center proteins. Furthermore, our Pt-TP data support this conclusion since it indicates that both the histidyl residues detected by the DPC-inhibition assay must be on surface-exposed protein segments. None of these amino acids, however, appear to be the first ligand to Mn in the photoactivation process, a role apparently played by Asp170 on D1 (*21*, *45*). However, the loss of processing of the D1 C-terminal extension (*5*) or mutation of histidine residues on the carboxyl end of the D1 protein does impair O_2 evolution capacity (*24*, *25*, *42*, *43*). While the exact role of these histidine residues in functional Mn binding is still speculative, there is an important function for histidine residues located near the carboxyl end of the D1, and perhaps the D2, protein at the catalytic site of the O_2-evolving complex.

ACKNOWLEDGMENT

We thank Dr. Nenad Kostic for directing our attention to Pt-TP and Professor Richard Debus for helpful comments on an earlier version of this paper.

REFERENCES

1. Lundblad, R. L., and Noyes, C. M. (1984) *Chemical Reagents for Protein Modification*, CRC Press Inc., Boca Raton, FL.
2. Prochaska, L. J., and Dilley, R. A. (1980) *Methods Enzymol. 69*, 502−510.
3. Tamura, N., Ikeuchi, M., and Inoue, Y. (1989) *Biochim. Biophys. Acta 973*, 281−289.
4. Seibert, M., Tamura, N., and Inoue, Y. (1989) *Biochim. Biophys. Acta 974*, 185−191.
5. Preston, C., and Seibert, M. (1989) *Photosynth. Res. 22*, 101−113.
6. Preston, C., and Seibert, M. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I, pp 925−928, Kluwer Academic Publishers, Dordrecht, The Netherlands.
7. Preston, C., and Seibert, M. (1991) *Biochemistry 30*, 9615−9624.
8. Preston, C., and Seibert, M. (1991) *Biochemistry 30*, 9625−9633.
9. Miles, E. W. (1977) *Methods Enzymol. 47*, 431−442.
10. Ratilla, E. M. A., Brothers, H. M., II, and Kostic, N. M. (1987) *J. Am. Chem. Soc. 109*, 4592−4599.
11. Brothers, H. M., II, and Kostic, N. M. (1988) *Inorg. Chem. 27*, 1761−1767.
12. Hsu, B.-D., Lee, J.-Y., and Pan, R.-L. (1987) *Biochim. Biophys. Acta 890*, 89−96.
13. Ghirardi, M. L., Lutton, T. W., and Seibert, M. (1998) *Biochemistry 37*, 13559−13566.

14. Ghirardi, M. L., Lutton, T. W., and Seibert, M. (1996) *Biochemistry 35*, 1820−1828.
15. Metz, J. G., and Seibert, M. (1984) *Plant Physiol. 76*, 829−832.
16. Tamura, N., Noda, K., Wakamatsu, K., Kamachi, H., Inoue, H., and Wada, K. (1997) *Plant Cell Physiol. 38*, 578−585.
17. Dunahay, T. G., Staehelin, L. A., Seibert, M., Ogilvie, P. D., and Berg, S. P. (1984) *Biochim. Biophys. Acta 764*, 179−183.
18. Zimmerman, J.-L., Boussac, A., and Rutherford, A. W. (1993) *Biochemistry 32*, 4831−4841.
19. Tang, X.-S., Sivaraja, M., and Dismukes, G. C. (1993) *J. Am. Chem. Soc. 115*, 2382−2389.
20. Tang, X.-S., Diner, B. A., Larsen, B. S., Gilchrist, M. L., Jr., Lorigan, G. A., and Britt, R. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 704−708.
21. Nixon, P. J., and Diner, B. A. (1992) *Biochemistry 31*, 942−948.
22. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6137−6149.
23. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6150−6157.
24. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1995) *Biochemistry 34*, 5839−5858.
25. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1995) *Biochemistry 34*, 5859−5882.
26. Kramer, D. M., Roffey, R. A., Govindjee, and Sayre, R. T. (1994) *Biochim. Biophys. Acta 1185*, 228−237.
27. Ono, T., and Inoue, Y. (1991) *Biochemistry 30*, 6183−6188.
28. Ono, T., and Inoue, Y. (1991) *FEBS Lett. 278*, 183−186.
29. Boussac, A., Zimmerman, J.-L., Rutherford, A. W., and Lavergne, J. (1990) *Nature 347*, 303−306.
30. Boussac, A., and Rutherford, A. W. (1995) *Biochim. Biophys. Acta 1230*, 195−201.
31. Roffey, R. A., Kramer, D. M., Govindjee, and Sayre, R. T. (1994) *Biochim. Biophys. Acta 1185*, 257−270.
32. Gilchrist, M. L., Ball, J. A., Randall, D. W., and Britt (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9545−9549.
33. Blubaugh, D. J., and Cheniae, G. M. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. 2, pp 361−364, Kluwer Academic Publishers, Dordrecht, The Netherlands.
34. Magnuson, A., and Andréasson, L.-E. (1997) *Biochemistry 36*, 3254−3261.
35. Mohanty, P., and Seibert, M. (1997) *Ind. J. Biochem. Biophys. 34*, 241−248.
36. Holshuh, K., Bottomley, W., and Whitfeld, P. R. (1984) *Nucleic Acids Res. 12*, 8819−8834.
37. Trebst, A. (1986) *Z. Naturforsch. 41C*, 240−245.
38. Ikeuchi, M., and Inoue, Y. (1988) *Plant Cell Physiol. 29*, 1233−1239.
39. Ikeuchi, M., and Inoue, Y. (1988) *FEBS Lett. 241*, 99−104.
40. Tae, G.-S., Black, M. T., Cramer, W. A., Vallon, O., and Bogorad, L. (1988) *Biochemistry 27*, 9075−9080.
41. Webber, A. N., Hird, S. M., Packman, L. C., Dyer, T. A., and Gray, J. C. (1989) *Plant Mol. Biol. 12*, 141−151.
42. Nixon, P. J., Chisholm, D. A., and Diner, B. A. (1992) in *Plant Protein Engineering* (Shewry, P., and Gutteridge, S., Eds) pp 93−141, Cambridge University Press, Cambridge, U.K.
43. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
44. Ambler, R. P. (1967) *Methods Enzymol. 11*, 155−166.
45. Boerner, R. J., Nguyen, A. P., Barry, B. A., and Debus R. J. (1992) *Biochemistry 31*, 6660−6672.

BI980359O