Plant Cell Physiol. 38(5): 578–585 (1997)

Involvement of Carboxyl Groups of the PSII Reaction Center Proteins in Photoactivation of the Apo-Water-Oxidizing Complex

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Involvement of residues of acidic amino acids in photoligation of manganese into the apo-water-oxidizing complex was investigated by use of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), a water-soluble carboxyl modifier. Treatment of Mn-depleted PSII membranes by EDC in the presence of nucleophiles induced a loss of photoactivation capability in the Mn complex and partial loss of capability of photooxidation of Mn^{2+}, but little decrease in the DCIP photoreduction supported by diphenylcarbazide. The inhibition of diphenylcarbazide-photooxidation by submicromolar Mn^{2+}, indicative of the intactness of high-affinity Mn-binding sites, was apparently abolished by EDC treatment. From amino acid quantitation analysis of D1 and D2 proteins and CP47 of the chemically-modified membranes, approximately three carboxyl groups of the D1 protein were found to be chemically-modified with EDC after removal of the functional Mn. These results suggest that acidic amino acids on the D1 protein are involved in photoactivation of the apo-water-oxidizing complex and probably in ligation of Mn to the water-oxidizing complex.

Key words: Chemical modifier — Manganese — Oxygen evolution — Photoactivation — Photosystem — Spinach.

In oxygenic organisms, manganese plays a central role in the water-oxidizing reaction to evolve oxygen. The tetra Mn atoms function in an accumulation of oxidizing equivalents generated by photoreactions, and are clustered within PSII intrinsic proteins (Amesz 1983, Debus 1992). EXAFS studies on PSII (Yachandra et al. 1987, McDermott et al. 1988, Penner-Hahn et al. 1990, Yachandra et al. 1993) have revealed that Mn-X (X=Mn, N or O) interactions with different distances occur in the Mn cluster; two-three (Penner-Hahn et al. 1990), or three-five (Prince et al. 1987, George et al. 1989) ≈2.7 Å Mn-Mn interactions, two ≈1.8 Å Mn-O or Mn-N interactions, two-four 1.9–2.1 Å Mn-O or Mn-N interactions and one ≈3.3 Å interaction. On the basis of these findings, a model has been presented that the Mn cluster consists of two di-μ_2-oxo dimers which are bridged by one mono-μ_2-oxo and two carboxylate bridges (Yachandra et al. 1993). According to this model, more than ten other ligands should be required.

On the basis of Mn coordination chemistry, possible candidates as ligands to the Mn complex have been proposed to be carboxyl-, alkoxo-, phenoxo- and imidazole-groups in amino acids (Pecoraro 1988). Precise identification of the proteins ligating functional Mn still remains to be done. However, considerable evidence has been accumulated that carboxyl groups and histidine residues on the D1 and D2 proteins are ligands to the Mn complex. From studies on site-directed mutations in cyanobacterium (Vermaas et al. 1990, Boerner et al. 1992, Debus 1992, Nixon and Diner 1992), potential ligands to the Mn complex have been proposed as follows: seven acidic amino acid residues (Asp-59, -61, -170, -342, Glu-65, -189, -333) and two histidine residues (His-190, -337) in the lumen side of the D1 protein (Boerner et al. 1992, Debus 1992, Nixon and Diner 1992, Chu et al. 1995), and Glu-69 (Vermaas et al. 1990, 1993) of the D2 protein. Recently, CP47 and CP43 have been considered to function in ligation of Mn (Carpenter et al. 1993, Gleiter et al. 1995). Furthermore, using combined techniques of photoligation of Mn^{2+} to the apo-water-oxidizing complex and chemical modification, other possible ligands to the Mn complex have been proposed (Seibert et al. 1989, Tamura et al. 1989a, 1992, Preston and Seibert 1991a, b). Treatment of diethyl pyrocarbonate (DEPC), a chemical modifier of histidine residues, caused a loss of photoactivation capability of PSII membranes depleted of functional Mn, and specifically modified histidine residues on the D1 protein (Tamura et al. 1989a). It was also found that chemical modification of carboxyl residues with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) abolishes photoactivation capability (Tamura et al. 1992). Preston and Seibert (1991a, b) also reported that chemical modification of the apo-Mn complex with EDC and DEPC causes partial loss of the high-affinity Mn-binding sites for binding of Mn^{2+}, respectively and concluded that carboxyl residues and histidine residues of the D1 protein such as His-337 function in binding and/or ligation

Abbreviations: CP47 and CP43, PSII chlorophyll binding proteins; DCIP, 2,6-dichlorophenol indophenol; DEPC, diethyl pyrocarbonate; DPC, 1,5-diphenylcarbazide; EDC,1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Q_A and Q_B, primary and secondary quinone acceptors on the reducing side of PSII; SML, sucrose monolaurate.

of Mn.

In this study, NH_2OH-treated PSII membranes were chemically modified with EDC in the presence of a nucleophile, and the effects of the treatment on photoactivation were investigated. The results obtained suggest that carboxyl groups on the D1 protein are involved in photoligation of Mn^{2+} to the apo-water-oxidizing complex.

Materials and Methods

The oxygen-evolving PSII membranes were obtained from spinach leaves as described in Radmer et al. (1986). Treatment of the membranes with NH_2OH was done as described in Tamura and Cheniae (1985). These preparations were used either directly or after storage at −80°C.

Chemical modification of PSII membranes by EDC was carried out at 20°C in buffer A (50 mM MES-NaOH, pH 6.5, 20 mM NaCl, 0.4 M sucrose) containing PSII membranes equivalent to 250 μg Chl ml^{-1}, 3–5 mM EDC and 1 M glycine or glycine ethyl ester, unless otherwise noted. The chemical modification involves reacting carboxyl groups of PSII membranes with EDC in the presence of a nucleophile such as glycine and glycine ethyl ester as follows;

$$\text{protein-COO}^- + {}^+\text{NH}_3\text{CH}_2\text{COO}^- \rightarrow \text{protein-CONHCH}_2\text{COO}^- \quad (1)$$

$$\text{protein-COO}^- + {}^+\text{NH}_3\text{CH}_2\text{COOC}_2\text{H}_5 \rightarrow \text{protein-CONHCH}_2\text{COOC}_2\text{H}_5 \quad (2)$$

(Lundblad 1991). A negative charge is conserved in eq. (1), while a negative charge is changed to the neutral in eq. (2). The reaction was stopped at a given time by an addition of 250 mM sodium acetate. The membranes were then washed three times in buffer A, and resuspended in the same buffer. The obtained EDC-treated PSII membranes were subjected to photoactivation as described in Tamura and Cheniae (1987). The PSII membranes (250 μg Chl ml^{-1}) were incubated with buffer A containing 1 mM $MnCl_2$, 50 mM $CaCl_2$ and 100 μM DCIP, at 25°C for 30 min under weak light (30 μE m^{-2} s^{-1}).

To obtain D1 and D2 proteins for amino acid analysis, PSII core complexes were prepared from PSII membranes and NH_2OH-treated ones, both of which were chemically-modified with EDC, as follows: PSII membranes (10 mg Chl) were incubated with 1% sucrose monolaurate (SML) in buffer B (20 mM MES-NaOH, pH 6.2, 20 mM NaCl) for 1 h at 4°C at 1 mg Chl ml^{-1} with stirring. The solubilized membranes were centrifuged at 40,000 × *g* for 15 min, and the resultant supernatant was loaded on an anionic exchange column (DEAE-Toyopearl 650S) equilibrated with buffer B containing 0.05% SML. The column was washed with 500 ml of buffer B containing 0.1% SML, and subjected to the gradient elution of NaCl (30–300 mM) in buffer B containing 0.1% SML. Two chlorophyll-enriched fractions were eluted at 100–130 and 150–170 mM NaCl, respectively. A first peak was found to contain the light-harvesting chlorophyll proteins, while a second one the PSII core complex consisting of D1 and D2 proteins and CP47 (Tamura et al. 1991). The second peak was concentrated with an Amicon ultrafiltration membrane (YM-10) and subjected to SDS-polyacrylamide gel electrophoresis (see lanes 6 and 7 in Fig. 2). SDS-PAGE was performed on a vertical 1-mm slab gel of 12.5% acrylamide containig 6 M urea. Following electrophoresis, the gel was equilibrated in a transfer buffer (25 mM Tris, pH 9.5, 40 mM norleucine, 20% methanol, 0.05% SML) for 15 min, and was then electroblotted on the polyvinylidene difluoride (PVDF) membrane for 1.5 h at 0.8 mA cm^{-2}. The PVDF membrane was incubated in 10 mM sodium borate (pH 8.0) containing 25 mM NaCl for 5 min to remove any excess glycine, rinsed in water and then stained in 0.1% Coomassie brilliant blue R-250 in 40% methanol and 15% acetic acid for 5 min. Excess dye was removed by a brief wash with water followed by destaining in 70% methanol. Areas of PVDF membranes containing D1 and D2 proteins and CP47 were excised. The membranes were anaerobically hydrolyzed in 6 M HCl including 0.05% phenol at 110°C for 24 h. After hydrolysis, the samples were dissolved in an equilibration buffer (Wako Pure Chemicals, Japan) and placed in a Hitachi amino acid analyzer.

O_2 evolution was determined in buffer A containing 1 mM potassium ferricyanide and 300 μM phenyl-*p*-benzoquinone, with a Clark-type oxygen electrode as described earlier (Tamura and Cheniae 1987). DCIP photoreduction was measured spectrophotometrically at 590 nm on the split mode with a Shimadzu spectrophotometer (UV-300). The reaction mixture basically contained 50 μM DCIP, 1 mM $MnCl_2$ or 500 μM diphenylcarbazide (DPC), and PSII membranes equivalent to 10 μg Chl ml^{-1} in buffer A, unless otherwise noted. The measurement of thermoluminescence component, A_T-band, was carried out as described in Ono and Inoue (1992).

Data analysis for obtaining the Michaelis-Menten constants was done by curve-fits to the Michaelis-Menten equation with a nonlinear curve-fitting Igor program (Wavemetrics, U.S.A.) on a Macintosh computer.

Results and Discussion

Figure 1 shows the effects of EDC treatment on DCIP photoreduction supported by Mn^{2+} or DPC and the photoactivation capability in NH_2OH-treated PSII membranes.

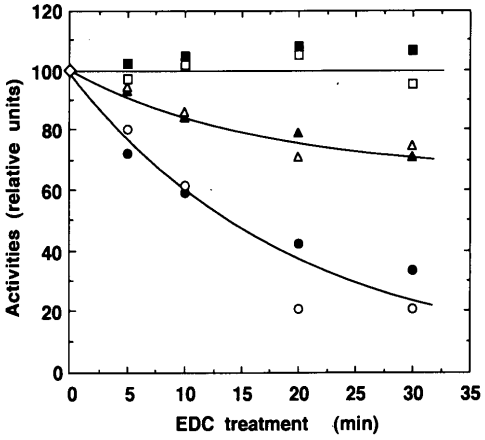

Fig. 1 Effects of EDC treatment on PSII electron transfer and photoactivation in NH_2OH-treated PSII membranes. Open and closed symbols were obtained when glycine and glycine ethyl ester were used as nucleophiles, respectively. Circles, photoactivation. Squares and triangles denote activities of DCIP photoreduction supported by DPC and Mn^{2+}, respectively. The activities of DCIP photoreduction donated by DPC and Mn^{2+} of unmodified NH_2OH-treated PSII membranes were 240 and 145 μmol DCIP (mg Chl)$^{-1}$ h^{-1}, respectively, and the restored O_2-evolving activity after photoactivation was 105 μmol O_2 (mg Chl)$^{-1}$ h^{-1}.

There was little difference in the effects of chemical modification of carboxyl groups on photoactivation and PSII donor activities when glycine and glycine ethyl ester, respectively, were used as nucleophiles. Within the time examined, the DCIP photoreduction supported by DPC was not affected by EDC treatment, while the DCIP photoreduction by Mn^{2+} decreased 30% compared to the control in 30 min-EDC-treated preparations. On the other hand, photoactivation capability decreased 70–80% after 30 min-EDC treatment. The time required for the half-maximum effect was 14 min, independent of the species of nucleophiles. The second-order rate constant was estimated as $13.2\ min^{-1}\ M^{-1}$.

In the absence of nucleophile in this reaction, a reaction intermediate, *O*-acyl isourea, reacts with amino groups in the surroundings to generate intermolecular or intramolecular crosslinking (Lundblad 1991). We studied whether nucleophiles such as glycine and glycine ethyl ester avoid secondary effects of the zero-length crosslinking in our conditions (Fig. 2). The modification in the absence of added nucleophile induced significant decrease in density of the bands for CP47, CP43, the 33 kDa extrinsic protein, and LHCP in NH_2OH-treated PSII membranes (Fig. 2, lane 3). Nucleophile at 50–200 mM still induced several bands in the high M_r region in a SDS-PAGE gel (data not shown), but nucleophile at 1 M did not (Fig. 2, lanes 4 and 5). There was little crosslinking in PSII core complexes isolated from NH_2OH-treated PSII membranes that were treated with EDC in the presence of 1 M glycine, and the D1 and D2 proteins were clearly separated in a gel (Fig. 2, lane 7).

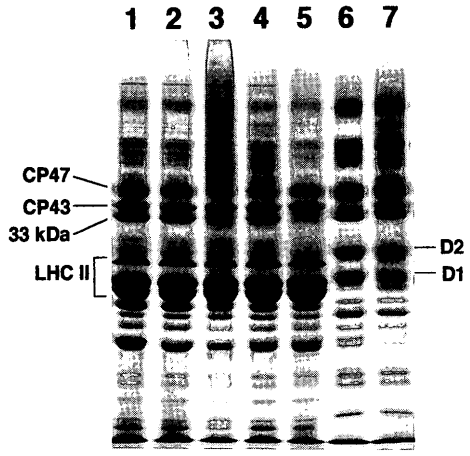

Fig. 2 SDS-PAGE gel on EDC-treated PSII membranes and PSII core complexes. Lane 1, PSII membranes; lane 2, NH_2OH-treated PSII membranes; lanes 3, NH_2OH-treated PSII membranes that were chemically-modified in the absence of any nucleophile; lanes 4 and 5, NH_2OH-treated PSII membranes that were chemically-modified in the presence of 1 M glycine and 1 M glycine ethyl ester respectively; lanes 6 and 7, PSII core complexes from PSII membranes and NH_2OH-treated PSII ones, respectively, both of which were chemically-modified in the presence of 1 M glycine.

Submicromolar Mn^{2+} given to PSII membranes depleted of functional Mn suppresses the photooxidation of DPC, acting in a competitive manner (Hsu et al. 1987, Tamura et al. 1989a) or a noncompetitive manner (Preston and Seibert 1991a) with respect to DPC. Hence, an inhibition constant (K_i) reflects an apparent dissociation constant for exogenous Mn^{2+} to the high-affinity Mn-binding sites. Table 1 shows that the suppression of DPC-photooxidation by Mn^{2+} was markedly decreased by EDC treatment. The value for unmodified NH_2OH-treated PSII membranes was 0.38 μM, which was close to the previously reported ones (Hsu et al. 1987, Tamura et al. 1989a). With an increasing extent of EDC treatment, the K_i value drastically increased with a decrease in photoactivation capability. EDC treatment prolonged to 60 min caused an almost complete loss of photoactivation capability and about a 10-fold increase in K_i. These results also indicate that the chemical modification of carboxyl groups on acidic amino acids by EDC decreases the affinity of Mn^{2+} to the high-affinity Mn-binding sites exposed to the lumen side.

In order to determine if EDC-treatment affects the reducing side as well as the oxidizing side of PSII, the effects of DCMU on the DCIP photoreduction in EDC-treated NH_2OH-PSII membranes were studied. Under our experimental conditions, approx. ten acidic amino acid residues were chemically modified on the D1 protein of NH_2OH-treated PSII membranes (see Table 3). DCMU is known to bind to the extra loop between helix IV and V of the D1 protein, which folds back over the Q_B site and possesses seven glutamic acids (Trebst 1986). The DCMU concentration dependences of the DCIP photoreduction were almost identical in all preparations examined (data not shown). The values of I_{50} (the concentration of DCMU that results in 50% inhibition) for the activity were 0.2 μM, independent of the degree of EDC treatment. From these results taken together, we suggest that EDC-treatment does not cause a

Table 1 Relationship between photoactivation capability and K_i values for Mn^{2+} in EDC-treated NH_2OH-PSII membranes

EDC treatment	K_i for Mn^{2+} (μM)	Regenerated VO_2 [μmol O_2 (mg Chl)$^{-1}$ h^{-1}]
None	0.38	96
5 min, Dark	2.1	40
60 min, Dark	3.1	8

The values of K_i for Mn^{2+} were obtained from the inhibition constants of added Mn^{2+} against the DCIP photoreduction activity supported by DPC as described in Kamachi et al. (1992). VO_2 denotes the oxygen evolving activities. Glycine was used as a nucleophile for the EDC-treatment.

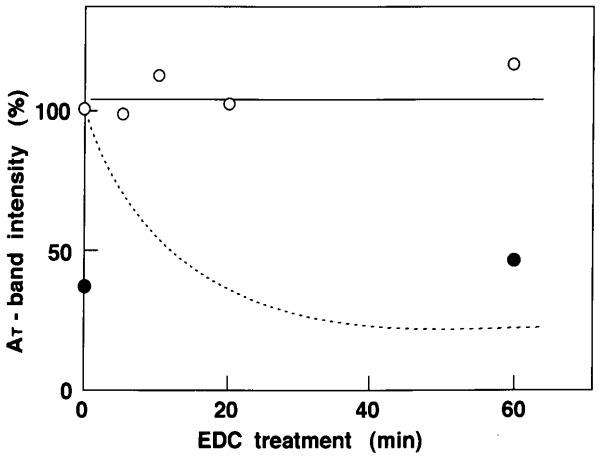

Fig. 3 Effects of EDC treatment on formation of the A_T-band in NH_2OH-treated PSII membranes. Open and closed circles depict the amplitudes of the A_T-band in the absence and presence of 1 mM Mn^{2+}. The broken line shows photoactivation capability against EDC treatment as described in Fig. 1. Glycine was used as a nucleophile for the EDC-treatment.

drastic change on the PSII-reducing side, but on the PSII-oxidizing side.

The thermoluminescence component denoted as the A_T-band, which was found by Ono and Inoue (1991a, b), is ascribed to arise from charge recombination between Q_A^- and an oxidized histidine in the PSII-oxidizing side. This component was reported to be abolished by DEPC treatment (Ono and Inoue 1991b). We examined whether the EDC treatment also affects the formation of the A_T-band (Fig. 3). As shown in Fig. 1, photoactivation capability decreased by 70% in 30 min-EDC treatment (broken line in Fig. 3). On the other hand, the amplitude of the A_T-band was not affected by the EDC treatment. When NH_2OH-treated PSII membranes were illuminated at −20°C in the presence of 1 mM Mn^{2+}, the intensity of the band decreased to 44% of that obtained in the absence of Mn^{2+}, due to the reduction of an oxidized histidine by exogenous Mn^{2+} (Ono and Inoue 1991b). The extent of the decrease in the intensity of A_T-band by Mn^{2+} was not significantly changed by EDC treatment for 60 min. These results thus exclude the possibility that EDC treatment modifies or affects the histidine residue which may oxidize and ligate Mn^{2+}. The site of chemical modification with EDC is probably located further from both the histidine in question and Y_Z, the primary electron donor to oxidized P680.

Table 2 shows the effects of EDC treatment on the O_2-evolving activity in PSII membranes and on photoactivation capability in NH_2OH-treated PSII membranes. There was little decrease in the O_2-evolving activity by EDC-treatment in PSII membranes (A in Table 2). On the other hand, NH_2OH-treated PSII membranes, which have no functional Mn, were susceptible to EDC treatment (Fig. 1, line 2 of B in Table 2). In contrast, PSII membranes that was treated by EDC and subsequently inactivated with NH_2OH showed almost complete photoactivation capability (C in Table 2). From these results, it is inferred that the site for chemical modification by EDC is largely protected by functional Mn and probably by the binding of Mn^{2+} to the high-affinity Mn-binding sites. A loss of photoactivation capability by EDC was significantly attenuated by the presence of 1 mM Mn^{2+} and 50 mM Ca^{2+} under weak light illumination as used for the photoactivation condition. We also found that exogenous Mn^{2+} and/or Ca^{2+}, in darkness, protected against EDC-inactivation of photoactivation capability to some extent (lines 3 and 4 of B in Table 2).

As previously reported (Tamura et al. 1989a), chemical modification of histidine residues by DEPC was prevented in the presence of Mn^{2+} under illumination but not in the dark. Such protection was not observed in the presence of any divalent cation except Mn^{2+}. The characteristic of two types of Mn-binding sites which are sensitive to either EDC or DEPC has already been reported by Preston

Table 2 Effects of various conditions of EDC-modification on the O_2-evolving activity in untreated PSII membranes and on the photoactivation capability in NH_2OH-treated PSII membranes

EDC treatment	VO_2 [μmol O_2 (mg Chl)$^{-1}$ h^{-1}]
A. PSII membranes	
None	632 (100)
40 min, Dark	661 (104)
B. NH_2OH-treated PSII membrane	
None	195 (100)
40 min, Dark	48 (25)
40 min, Dark, $+Mn^{2+}$	93 (48)
40 min, Dark, $+Mn^{2+}/+Ca^{2+}$	110 (56)
40 min, Light, $+Mn^{2+}/+Ca^{2+}$ (10 min-preillumination)	162 (83)
C. PSII membranes	
40 min, Dark and NH_2OH-treated	190 (97)

In A, PSII membranes were treated with 5 mM EDC plus 1 M glycine for 40 min in darkness, washed and then assayed for their O_2-evolving activities. In B, NH_2OH-treated PSII membranes were chemically-modified in the presence and absence of 1 mM Mn^{2+}/50 mM Ca^{2+} either in darkness or under weak light preceded by 10 min-preillumination, and then photoactivated after two washes. In C, PSII membranes that were chemically-modified as described above were treated with 4 mM NH_2OH, followed by photoactivation after two washes. Numbers in parentheses of B and C denote the relative O_2-evolving activities restored by photoactivation, which are normalized to the activity obtained with unmodified NH_2OH-PSII membranes. Glycine was used as a nucleophile for the EDC-treatment.

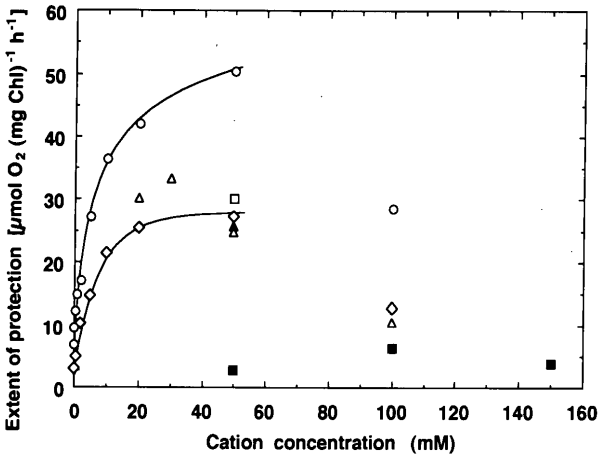

Fig. 4 Protection by divalent cations and sodium against EDC-inactivation in darkness. Extent of protection was calculated from the difference of O_2-evolving activities restored by photoactivation between preparations chemically-modified in the presence and absence of the following cations at a given concentration: Mn^{2+} (○), Ca^{2+} (◇), Mg^{2+} (△), Ba^{2+} (▲), Sr^{2+} (□) and Na^{+} (■). Lines represent curve-fits to the Michaelis-Menten equation of Mn^{2+}- and Ca^{2+}-protection of photoactivation. The residual O_2-evolving activity was 30 μmol O_2 (mg Chl)$^{-1}$ h^{-1} in NH_2OH-treated PSII membranes. After photoactivation of the NH_2OH-treated PSII membranes and the EDC-treated NH_2OH-PSII membranes, O_2-evolving activities were obtained at 108 and 41 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, respectively. Glycine was used as a nucleophile for the EDC-treatment.

and Seibert (1991a, b): By using the extent of non-competitive inhibition by Mn^{2+} of DPC-photooxidation, as indicative of the relative number of Mn-binding sites, they concluded that EDC modifies only half of the high affinity Mn-binding sites insensitive to DEPC. Thus, we studied the effects of dark incubation with divalent cations on EDC-modification of NH_2OH-treated PSII membranes (Fig. 4). The effectiveness of divalent cations to prevent EDC-inactivation was almost the same, except for Mn^{2+}. The extent of the protection by Mn^{2+} reached about 80% of the protection obtained under the photoactivation condition, whereas those by other divalent cations such as Ca^{2+} and Mg^{2+} only reached 40–50%. The maximum protection was obtained at 50 mM with each cation. However, the presence of monovalent cation at 150 mM, equivalent to 50 mM $MnCl_2$ on the basis of ionic strength, did not provide any protection from EDC-inactivation, indicating that protection from EDC attack was induced by divalent-specific binding, not by electrostatic screening of negative charges on the PSII membranes. From curve-fitting analysis, values of apparent K_m for Mn^{2+}-protection were estimated to be 0.05 and 7.5 mM, respectively. In contrast, Ca^{2+}-protection against EDC treatment showed a monophasic Michaelis-Menten-type dependence with K_m of 5.5 mM. The lower K_m value (0.05 mM) obtained for protection by Mn^{2+} that accounted for approximately 20% of the total Mn^{2+}-protection in darkness, was close to K_m values (0.05 mM) (Tamura et al. 1989a) or dissociation constants (0.04–0.05 mM) (Ono and Inoue 1983, Miller and Brudvig 1989) of Mn^{2+} for photoactivation. However, binding of Mn^{2+} to the high-affinity Mn-binding sites, that show K_m and inhibition constants less than 1 μM, has been detected by the photodecomposition of H_2O_2 in the presence of Mn^{2+} (Boussac et al. 1986, Inoue and Wada 1987), a competitive or noncompetitive inhibition of Mn^{2+} for DPC-supported DCIP photoreduction (Hsu et al. 1987, Tamura et al. 1989a for a competitive inhibition, and Preston and Seibert 1991a for a noncompetitive inhibition) or Mn^{2+}-supported chlorophyll fluorescence (Klimov et al. 1982). As suggested by Miller and Brudvig (1990) and by Blubaugh and Cheniae (1990), this discrepancy may be explained by the assumption that once oxidized to Mn^{3+}, Mn would be much more tightly bound to the Mn-binding sites: the dissociation constant then estimated could reflect the rate-limiting reaction, which is turnover and interaction of photogenerated Mn^{3+} by exogenous Mn^{2+} at its binding sites. Therefore, binding of Mn^{2+} to the high-affinity Mn-binding sites even in the dark is likely to protect carboxyl groups from the attack of EDC to some extent.

The higher K_m values for Mn^{2+}- and Ca^{2+}-protection were in the same order as those for Ca^{2+}-requiring S-state transitions in PSII membranes lacking of PSII extrinsic proteins (Boussac et al. 1986, Cammarata and Cheniae 1987). Divalent cations such as Ca^{2+}, Mg^{2+}, Ba^{2+} and Sr^{2+} were reported to competitively inhibit binding of Mn^{2+} to the endogenous Mn-binding sites, resulting in a decrease in the yield of photoligation of Mn^{2+} (Tamura et al. 1989b). However, it is still not clear how such divalent cations, at high concentrations, protect carboxyl groups from EDC attack specifically.

In order to ascertain which protein(s) is (are) specifically modified by EDC, we investigated the amino acid composition of D1 and D2 proteins and CP47 of PSII membranes and NH_2OH-treated PSII membranes, all of which were chemically-modified for 40 min. In this experiment, we adopted EDC-treated PSII membranes as the control that retained functional Mn, since the O_2-evolving activity was not decreased by EDC treatment in PSII membranes (A in Table 2) and a complete protection against EDC-inactivation in photoactivation of NH_2OH-treated PSII membranes was not observed (B in Table 2). According to eq. (1) described in Materials and Methods, an increased glycine content by EDC treatment corresponds to the maximum number of chemically-modified sites in each protein: we can not exclude a possibility that the successive addition of glycine(s) to the modified carboxyl residues occurs within time examined. The difference of EDC-modified sites in each protein between the EDC-treated PSII membranes and the EDC-treated NH_2OH-PSII membranes is con-

Table 3 Amino acid analyses of EDC-modified D1 and D2 proteins and CP47

Amino acid	D1 protein				D2 protein				CP47			
	A	B		B−A	A	B		B−A	A	B		B−A
Asx	25.8	25.7	(29)	−0.1	26.4	25.7	(27)	−0.7	29.4	29.4	(34)	0
Thr	13.7	12.8	(16)	−0.9	19.6	18.2	(20)	−1.4	22.0	22.5	(25)	+0.5
Ser	23.5	22.3	(26)	−1.2	20.7	19.4	(18)	−1.3	29.7	29.7	(34)	0
Glx	29.9	29.0	(26)	−0.9	34.5	33.0	(27)	−1.5	38.5	38.1	(34)	−0.4
Gly	39.6	41.6	(32)	+2.0	40.2	39.3	(31)	−0.9	66.2	66.0	(61)	−0.2
Ala	31.9	31.6	(33)	−0.3	37.3	36.5	(39)	−0.8	38.5	38.6	(39)	+0.1
Val	19.0	19.0	(21)	0	23.2	22.7	(22)	−0.5	35.4	35.8	(40)	+0.4
Ile	29.6	29.0	(30)	−0.6	14.6	14.8	(13)	+0.2	25.8	26.2	(30)	+0.4
Leu	31	31	(31)	0	41	41	(41)	0	39	39	(39)	0
Tyr	11.6	11.2	(12)	−0.4	9.7	9.3	(8)	−0.4	15.0	15.8	(17)	+0.8
Phe	20.8	20.3	(26)	−0.5	32.2	32.6	(39)	+0.4	32.2	32.0	(41)	−0.2
His	6.4	5.8	(10)	−0.6	5.2	5.4	(8)	+0.2	12.2	11.9	(14)	−0.3
Arg	13.9	12.3	(15)	−1.6	15.5	15.6	(15)	+0.1	23.5	25.9	(29)	+2.4

PSII core complexes from the EDC-treated PSII membranes and the EDC-treated NH_2OH-PSII membranes were subjected to SDS-PAGE. Following electrophoresis, the gel was electroblotted on PVDF membrane, of which areas containing stained proteins corresponding to D1 and D2 proteins and CP47 were excised. The membranes were hydrolyzed and then placed in an amino acid analyzer. See Materials and Methods for detail. A, PSII core complexes from the EDC-treated PSII membranes; B, PSII core complexes from the EDC-treated NH_2OH-PSII membranes. Asx and Glx denote Asp+Asn and Glu+Gln, respectively. Numbers indicate the experimental values (mol (mol of protein)$^{-1}$) of amino acids of D1 and D2 proteins and CP47, while those in parentheses the numbers of amino acids of the proteins of spinach obtained from data base (Zurawski et al. 1982, Holschuh et al. 1984, Morris and Herrmann 1984). The values in the Table were obtained from three independent experiments and errors were within ±3%. Glycine at 1 M was used as a nucleophile for the EDC-treatment.

sidered to be equivalent to the number of carboxyl groups involved in ligation of the functional Mn. Table 3 shows the amino acid compositions of the D1 and D2 proteins and CP47 in two preparations, which are normalized to the Leu contents. Of the species of amino acids observed, Glx, Leu, His and Phe, as well as Gly, had some discrepancies between experimental and predicted numbers. However, the obtained numbers of each amino acid of three proteins in question were in quite good agreement comparing the two types of EDC-treated membranes, except for glycine content in the D1 protein. The differences in glycine content between two EDC-treated preparations, denoted by B-A in Table 3, were +2.0, −0.9 and −0.2 mol mol^{-1} of protein in D1 and D2 proteins and CP47, respectively. There were also some changes in other amino acids such as −1.2 of Ser, −1.6 of Arg for the D1 protein, −1.4 of Thr, −1.3 of Ser, −1.5 of Glx for the D2 protein, and +2.4 of Arg for CP47. When amino acid contents were normalized to the contents of Val and Ala, the differences of glycine content were +2.2 and +2.5 in the D1 protein, +0.2 and +0.2 in the D2 protein, −1.0 and −0.3 in CP47, respectively. Considering this variance in the obtained data, we found a marked difference in glycine content in the D1 protein, but not in the D2 protein and CP47. Here, we could assume that only 75% of the total acidic amino acids of ligands are actually modified, on the basis of the result that 40 min-EDC-treatment caused approximately 75% loss of photoactivation capability of NH_2OH-treated PSII membranes under our experimental conditions (B in Table 2). By use of this assumption and the value of 2.0 mol mol^{-1} for the specifically modified carboxyl groups, the acidic amino acids on the D1 protein which are involved in photoactivation and probably contribute to ligands for Mn ions may be estimated to be less than 2.7 mol mol^{-1} of D1 protein.

Several groups have studied the site-directed mutations of the D1 and D2 proteins, which are constructed in the cyanobacterium *Synechocystis sp.* PCC 6803. Mutations of seven acidic amino acids (Asp-59, -61, -70 and -342, Glu-65, -189 and -333) and three histidines (His-190, -332 and -337) of the D1 protein abolished or slowed photoautotrophic growth (Boerner et al. 1992, Debus 1992, Chu et al. 1994, 1995, Whitelegge et al. 1995). Also, the mutation at Glu-69 on the D2 protein abolished or modified the photoautotrophic growth (Vermaas et al. 1990). The estimated values of the acidic amino acids in the D1 protein required for photoligation of Mn^{2+} in this study are less than those obtained from site-directed mutagenesis studies. However, it is not clear whether an acidic amino acid(s) on the D2 protein is (are) involved in ligation of functional Mn, based on the accuracy of amino acid analysis in this study. To obtain more accurate numbers of carboxyl groups functioning in ligands for Mn, the method for determining the modified sites described in this study

should be refined. On this line, we are now working on quantifying the numbers of specifically-modified sites in the fragments that are produced by digestion of the D1 and D2 proteins with proteases or chemicals.

The authors thank Dr. T.-A. Ono of RIKEN for measurement of thermoluminescence and fruitful discussion and Ms. T. Tanaka, K. Nomiyama and T. Shibata of Fukuoka Women's University for their helpful technical support. Thanks are also due to Drs. S.M. Theg and A. Stemler of University of California, Davis for critical reading of the manuscript. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education and Culture of Japan.

References

Amesz, J. (1983) The role of manganese in photosynthetic oxygen evolution. *Biochim. Biophys. Acta* 726: 1-12.

Blubaugh, D.J. and Cheniae, G.M. (1990) Kinetics of photoinhibition in hydroxylamine-extracted photosystem II membranes: relevance to photoactivation and sites of electron donation. *Biochemistry* 29: 5109-5118.

Boerner, R.J., Nguyen, A.P., Barry, B.A. and Debus, R.J. (1992) Evidence from directed mutagenesis that aspartate 170 of the D1 polypeptide influences the assembly and/or stability of the manganese cluster in the photosynthetic water-splitting complex. *Biochemistry* 31: 6660-6672.

Boussac, A., Picaud, M. and Etienne, A.C. (1986) Effect of potassium iridic chloride on the electron donation by Mn^{2+} to photosystem II particles. *Photobiochem. Photobiophys.* 10: 201-211.

Cammarata, K. and Cheniae, G.M. (1987) Studies on 17, 24 kD depleted photosystem II membranes. I. Evidence for high and low affinity calcium sites in 17, 24 kD depleted PSII membranes from wheat versus spinach. *Plant Physiol.* 84: 587-595.

Carpenter, S.D., Ohad, I. and Vermaas, W.F.J. (1993) Analysis of chimeric spinach/cyanobacterial CP43 mutants of *Synechocystis sp.* PCC-6803—The chlorophyll-protein CP43 affects the water-splitting system of photosystem-II. *Biochim. Biophys. Acta* 1144: 204-212.

Chu, H.A., Nguyen, A.P. and Debus, R.J. (1994) Site-directed photosystem II mutants with perturbed oxygen-evolving properties. 1. Instability or inefficient assembly of the manganese cluster in vivo. *Biochemistry* 33: 6137-6149.

Chu, H.A., Nguyen, A.P. and Debus, R.J. (1995) Amino acid residues that influence the binding of manganese or calcium to photosystem II. 1. The lumenal interhelical domains of the D1 polypeptide. *Biochemistry* 34: 5839-5858.

Debus, R.J. (1992) The manganese and calcium ions of photosynthetic oxygen evolution. *Biochim. Biophys. Acta* 1102: 269-352.

George, G.N., Prince, R.C. and Cramer, S.P. (1989) The Mn binding site of the photosynthetic water-splitting enzyme. *Science* 243: 789-791.

Gleiter, H.M., Haag, E., Shen, J.R., Eaton-Rye, J.J., Seeliger, A.G., Inoue, Y., Vermaas, W.F.J. and Renger, G. (1995) Involvement of the CP47 protein in stabilization and photoactivation of a functional water-oxidizing complex in the cyanobacterium *Synechocystis sp.* PCC 6803. *Biochemistry* 34: 6847-6856.

Holschuh, K., Bottomley, W. and Whitfeld, P.R. (1984) Structure of the spinach chloroplast genes for the D2 and 44 kDa reaction-center proteins of photosystem II and $tRNA^{Ser}$ (UGA). *Nucl. Acids Res.* 12: 8819-8834.

Hsu, B.-D., Lee, J.-Y. and Pan, R.-L. (1987) The high affinity binding site for manganese on the oxidizing side of photosystem II. *Biochim. Biophys. Acta* 890: 89-96.

Inoue, H. and Wada, T. (1987) Requirement of manganese for electron donation of hydrogen peroxide in photosystem II reaction center complex. *Plant Cell Physiol.* 28: 767-773.

Kamachi, H., Tamura, N. and Inoue, H. (1992) Putative second binding site of DCMU on the oxidizing side of photosystem II in photosystem II membranes depleted of functional Mn. *Plant Cell Physiol.* 33: 437-443.

Klimov, V.V., Allakhverdiev, S.I., Shuvalov, V.A. and Krasnovsky, A.A. (1982) Effect of extraction and re-addition of manganese on light reactions of photosystem-II preparations. *FEBS Lett.* 148: 307-312.

Lundblad, R.L. (1991) *In* Chemical Reagents for Protein Modification (2nd edition). pp. 267-286. CRC Press, Inc., Boca Raton.

McDermott, A.E., Yachandra, V.K., Guiles, R.D., Cole, J.L., Dexheimer, S.L., Britt, R.D., Sauer, K. and Klein, M.P. (1988) Characterization of the manganese O_2-evolving complex and the iron-quinone acceptor complex in photosystem II from a thermophilic cyanobacterium by electron paramagnetic resonance and X-ray absorption spectroscopy. *Biochemistry* 27: 4021-4031.

Miller, A.-F. and Brudvig, G.W. (1989) Manganese and calcium requirements for reconstitution of oxygen-evolution activity in manganese-depleted photosystem II membranes. *Biochemistry* 28: 8181-8190.

Miller, A.F. and Brudvig, G.W. (1990) Electron-transfer events leading to reconstitution of oxygen-evolution activity in manganese-depleted photosystem II membranes. *Biochemistry* 29: 1385-1392.

Morris, J. and Herrmann, R.G. (1984) Nucleotide sequence of the gene for the P680 chlorophyll *a* apoprotein of the photosystem II reaction center from spinach. *Nucl. Acids Res.* 12: 2837-2850.

Nixon, P.J. and Diner, B.A. (1992) Aspartate 170 of the Photosystem II reaction center polypeptide D1 is involved in the assembly of the oxygen-evolving cluster. *Biochemistry* 31: 942-948.

Ono, T.-A. and Inoue, Y. (1983) Requirement of divalent cations for photoactivation of the latent water-oxidation system in intact chloroplasts from flashed leaves. *Biochim. Biophys. Acta* 723: 191-201.

Ono, T.-A. and Inoue, Y. (1991a) Biochemical evidence for histidine oxidation in photosystem II depleted of the Mn-cluster for O_2-evolution. *FEBS Lett.* 278: 183-186.

Ono, T.-A. and Inoue, Y. (1991b) A possible role of redox-active histidine in the photoligation of manganese into a photosynthetic O_2-evolving enzyme. *Biochemistry* 30: 6183-6188.

Ono, T.-A. and Inoue, Y. (1992) Localization in photosystem II of the histidine residue putatively responsible for thermoluminescence A_T-band as probed by trypsin accessibility. *Biochim. Biphys. Acta* 1099: 185-192.

Pecoraro, V.L. (1988) Structural proposals for the manganese centers of the oxygen evolving complex: an inorganic chemist's perspective. *Photochem. Photobiol.* 48: 249-264.

Penner-Hahn, J.E., Fronko, R.M., Pecoraro, V.L., Yocum, C.F., Betts, S.D. and Bowlby, N.R. (1990) Structural characterization of the Mn sites in the photosynthetic oxygen evolving complex using X-ray absorption spectroscopy. *J. Amer. Chem. Soc.* 112: 2549-2557.

Preston, C. and Seibert, M. (1991a) The Carboxyl modifier 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide (EDC) inhibits half of the high-affinity Mn-binding site in photosystem II membrane fragments. *Biochemistry* 30: 9615-9624.

Preston, C. and Seibert, M. (1991b) Protease treatments of photosystem II membrane fragments reveal that there are four separate high-affinity Mn-binding sites. *Biochemistry* 30: 9625-9633.

Prince, R.C., Cramer, S.P. and George, G.N. (1987) Manganese cluster of the water-splitting enzyme. *In* Current Research in Photosynthesis. Edited by Baltscheffsky, M. Vol. I. pp. 685-692. Kluwer Academic Publishers, Dordrecht.

Radmer, R., Cammarata, K., Tamura, N., Ollinger, O. and Cheniae, G.M. (1986) Deletion of photosystem 2 extrinsic proteins. I. Effects on O_2 and N_2 flash yields and steady state O_2 evolution. *Biochim. Biophys. Acta* 850: 21-32.

Seibert, M., Tamura, N. and Inoue, Y. (1989) Lack of photoactivation capacity in *Scenedesmus obliquus* LF-1 results from loss of half the high-affinity manganese-binding site. Relationship to the unprocessed D1 protein. *Biochim. Biophys. Acta* 974: 185-191.

Tamura, N. and Cheanie, G.M. (1985) Effects of photosystem II extrinsic proteins on microstructure of the oxygen-evolving complex and its reactivity to water analogs. *Biochim. Biophys. Acta* 809: 245-259.

Tamura, N. and Cheniae, G.M. (1987) Photoactivation of the water-oxidizing complex in photosystem II membranes depleted of Mn and extrinsic proteins. I. Biochemical and kinetic characterization. *Biochim. Biophys. Acta* 890: 179-194.

Tamura, N., Ikeuchi, M. and Inoue, Y. (1989a) Assignment of histidine residues in D1 protein as possible ligands for functional manganese in photosynthetic water-oxidizing complex. *Biochim. Biophys. Acta* 973: 291-299.

Tamura, N., Inoue, Y. and Cheniae, G.M. (1989b) Photoactivation of the water-oxidizing complex in photosystem II membranes depleted of Mn, Ca and extrinsic proteins. II. Studies on the functions of Ca^{2+}. *Biochim. Biophys. Acta* 976: 173–181.

Tamura, N., Kamachi, H., Hokari, N., Masumoto, H. and Inoue, H. (1991) Photoactivation of the water-oxidizing complex of photosystem II core complex depleted of functional Mn. *Biochim. Biophys. Acta* 1060: 51–58.

Tamura, N., Tanaka, T., Wakamatsu, K., Inoue, H. and Wada, K. (1992) Possible role of carboxyl groups in photoactivation of the apo-water oxidizing complex. *In* Research in Photosynthesis. Edited by Murata, N. Vol. II. pp. 405–408. Kluwer Academic Publishers, Dordrecht.

Trebst, A. (1986) Topology of the plastoquinone and herbicide binding peptides of photosystem II in the thylakoid membrane. *Z. Naturforsch.* 41c: 240–245.

Vermaas, W., Charite, J. and Shen, G. (1990) Glu-69 of the D2 protein in photosystem II is a potential ligand to Mn involved in photosynthetic oxygen evolution. *Biochemistry* 29: 5325–5332.

Vermaas, W.F.J., Styring, S., Schroder, W.P. and Andersson, B. (1993) Photosynthetic water oxidation—The protein framework. *Photosynth. Res.* 38: 249–263.

Whitelegge, J.P., Koo, D., Diner, B.A., Domian, I. and Erickson, J.M. (1995) Assembly of the photosystem II oxygen-evolving complex is inhibited in psbA site-directed mutants of *Chlamydomonas reinhardtii*—Aspartate 170 of the D1 polypeptide. *J. Biol. Chem.* 270: 225–235.

Yachandra, V.K., DeRose, V.J., Latimer, M.J., Mukerji, I., Sauer, K. and Klein, M.P. (1993) Where plants make oxygen: a structural model for the photosynthetic oxygen-evolving manganese center. *Science* 260: 675–679.

Yachandra, V.K., Guiles, R.D., McDermott, A., Cole, J., Britt, R.D., Dexheimer, S.L., Sauer, K. and Klein, M.P. (1987) Comparison of the structure of the manganese complex in the S_1 and S_2 states of the photosynthetic O_2-evolving complex: an X-ray absorption spectroscopy study. *Biochemistry* 26: 5974–5981.

Zurawski, G., Bohnert, H.J., Whitfeld, P.R. and Bottomley, W. (1982) Nucleotide sequence of the gene for the M_r 32,000 thylakoid membrane protein from *Spinacia oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of M_r 38,950. *Proc. Natl. Acad. Sci. USA* 79: 7699–7703.

(Received October 21, 1996; Accepted March 3, 1997)