Carboxylate Groups on the Manganese-Stabilizing Protein Are Required for Its Efficient Binding to Photosystem II†

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ABSTRACT: The effects of the modification of carboxylate groups on the manganese-stabilizing protein of photosystem II were investigated. Carboxylate groups (including possibly the C-terminus) on the manganese-stabilizing protein were modified with glycine methyl ester in a reaction facilitated by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide. The manganese-stabilizing protein that was modified while associated with NaCl-washed photosystem II membranes contained 1−2 modified carboxylates, whereas the protein that was modified while free in solution contained 4 modified carboxylates. Both types of modified protein could reconstitute oxygen evolution at high manganese-stabilizing protein to photosystem II reaction center ratios. However, the protein that had been modified in solution exhibited a dramatically altered binding affinity for photosystem II. No such alteration in binding affinity was observed for the protein that had been modified while associated with the photosystem. Mapping of the sites of modification was carried out by trypsin and *Staphylococcus* V8 protease digestion of the modified proteins and analysis by matrix-assisted laser desorption/ionization mass spectrometry. These studies indicated that the domains ^{157}D−^{168}D and ^{212}E−^{247}Q (C-terminus) are labeled only when the manganese-stabilizing protein is modified in solution. Modified carboxylates in these domains are responsible for the altered binding affinity of this protein for the photosystem.

In higher plants and cyanobacteria at least six intrinsic proteins appear to be required for oxygen evolution by photosystem II (PS II)[1] (*1*−*3*). These are CP 47, CP 43, the D1 and D2 proteins, and the α and β subunits of cytochrome b_{559}. Insertional inactivation or deletion of the genes for these components results in the complete loss of oxygen evolution activity. Additionally, a number of low molecular mass components appear to be associated with PS II (*4*, *5*), although the functions of these proteins remain obscure. While PS II complexes containing only these components can evolve oxygen, they do so at low rates (about 25−40% of control), are extremely susceptible to photoinactivation, and require high, nonphysiological levels of calcium and chloride for maximal activity (*1*, *3*).

In higher plants three extrinsic proteins, with apparent molecular masses of 33, 24, and 17 kDa, are required for high rates of oxygen evolution at physiological inorganic cofactor concentrations. The 33 kDa component has been termed the manganese-stabilizing protein (MSP) due to its stabilization of the manganese cluster during exposure to low chloride concentrations or to exogenous reductants. In cyanobacteria, only the MSP is present, with the functions of the 23 and 17 kDa proteins possibly being provided by cytochrome c_{550} and a 12 kDa protein (*6*). These three extrinsic components apparently interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes. Despite a rather large literature examining the extrinsic proteins, in general, and the MSP, in particular, relatively little is known of the structure or functional roles played by these components within the photosystem.

The amino acid residues present on the MSP that are required for its interaction with PS II have not been identified. Cross-linking studies using the water-soluble carbodiimide EDC have indicated that the MSP interacts with CP 47 and possibly other components of PS II via a charge-pair interaction (*7*, *8*). Seidler (*9*) examined the role of carboxylates on the binding of the MSP to PS II. Differential activation of the carboxylates on the MSP and on the intrinsic proteins of PS II was performed with EDC and sulfo(*N*-hydroxy)succinimide. This author concluded that all of the carboxyl groups involved in cross-link formation between the intrinsic PS II proteins and the MSP are present on the extrinsic component. Alternatively, Miura et al. (*10*) modified the aspartyl and glutamyl residues on the MSP with glycine methyl ester (GME) in an EDC-mediated reaction. These authors reported the modification of more than eight

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[1] Abbreviations: chl, chlorophyll; DCBQ, 2,6-dichlorobenzoquinone; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GME, glycine methyl ester; LiDS, lithium dodecyl sulfate; MALDI, matrix-assisted laser desorption/ionization; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein; PS, photosystem; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoracetic acid; VP, 4-vinylpyridine.

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unidentified carboxylate groups on the MSP. After modification, these authors found that, at a high GME-modified MSP to PS II reaction center ratio, the modified protein could still bind to PS II and fully reconstitute oxygen evolution activity. They concluded that carboxylate groups on the MSP do not participate in the binding of the MSP to PS II and hypothesized that basic residues on the MSP were responsible for its binding. Thus, the studies by Seidler (*9*) and Miura et al. (*10*) come to precisely opposite conclusions concerning the role of carboxylate residues in the binding of the MSP to PS II.

In this study, we have reexamined the role of the carboxylates on the MSP in the binding of this component to PS II. We have found that the modification of carboxylates dramatically affects the ability of the MSP to bind to the photosystem although the modification does not substantially affect oxygen-evolving activity at saturating protein concentrations. We have also mapped the regions on the MSP that contain the modified carboxylate groups.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as previously described (*11*). Chl concentration was measured by the method of Arnon (*12*). Oxygen-evolving PS II membranes were prepared by the method of Berthold et al. (*13*) as modified by Ghanotakis and Babcock (*14*). Typical preparations exhibited a chl *a*/*b* ratio of 1.9−2.0 and an oxygen evolution rate of about 500 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}. The extrinsic 24 and 17 kDa proteins were removed from the PS II membranes by incubation for 1 h in 1.0 M NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0 at a chl concentration of 1.0 mg/mL and then washing again in the same buffer.

Labeling of the MSP with GME either in solution or on the PS II membranes was performed essentially according to Miura et al. (*10*). This reaction, which is facilitated by the presence of EDC, leads to the modification of aspartyl and glutamyl residues. Additionally, the C-terminus of the MSP may be modified by this procedure. The EDC first reacts with free carboxyl groups on the MSP, forming an *O*-acylisourea. This activated carboxyl then undergoes a nucleophilic attack by the amino group of the GME with the concomitant displacement of a urea derived from the carbodiimide (*15*).

Purified MSP (0.5 mg/mL) (*16*) was suspended in 20 mM sodium/potassium phosphate, pH 6.6, 100 mM GME, and 2 mM EDC overnight at 24 °C. The protein solution was then brought to 100 mM NaCl and the labeling reagents were removed by several rounds of centrifugal ultrafiltration. The MSP that was associated with NaCl-washed PS II membranes was labeled by suspension of the membranes in 300 mM sucrose, 15 mM NaCl, 10 mM $MgCl_2$, 50 mM Mes−NaOH, pH 6.0, 100 mM GME, and 2 mM EDC overnight at 24 °C. The membranes were then washed twice with 100 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove the labeling reagents. The modified MSP was then removed by treatment with 1.0 M $CaCl_2$, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, for 1 h at a chl concentration of 1.0 mg/mL at 4 °C. After centrifugation to remove the PS II membranes, the protein solution was dialyzed overnight against 10 mM Mes−NaOH, pH 6.0, filtered through a 0.3 μm filter to remove residual membrane fragments, and concentrated by centrifugal ultrafiltration.

Time-of-flight mass spectrometry was performed on the MSP at either the Wistar Protein Microchemistry Laboratory (Philadelphia, PA) or the Louisiana State University Mass Spectrometry Facility. Protein samples were diluted to 1−5 pmol/μL with 0.1% TFA and mixed with a matrix. A saturated solution of sinapinic acid was used for the intact MSP. Saturated solutions of either cyano-4-hydroxycinnamic acid or sinapinic acid were used for the proteolytic peptides (see below). The samples were analyzed on either a PerSpective Biosystems Voyager Biospectrometry Workstation (Wistar Institute) or a Bruker Daltronics Proflex III instrument (LSU). Two internal mass standards, carbonic anhydrase (29.024 kDa) and bovine serum albumin (66.431 kDa), were used for instrument calibration for the intact MSP. Mass spectra for the proteolytic fragments were calibrated by using external standardization. The MALDI mass spectra were analyzed with the GPMAW Program version 3.0 (Lighthouse Data, Denmark).

Reconstitution experiments were performed with unmodified MSP, MSP that had been modified in solution, and MSP that had been modified while associated with the PS II membrane. For these experiments, PS II membranes were washed twice with 1.0 M $CaCl_2$, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove the MSP. The membranes were then washed twice with, and resuspended in, 100 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove the residual $CaCl_2$. The MSP-depleted membranes (50 μg/mL chl) were then incubated with various concentrations of the different MSP preparations.

For the examination of the ability of the various modified MSP preparations to reconstitute oxygen evolution activity, the reconstituted membranes were incubated for 1 h on ice and then assayed by oxygen polarography at 24 °C in 100 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, in the presence of 1 mM DCBQ at 2000 μmol of photons m^{-2} s^{-1}.

For the direct analysis of protein binding after incubation with the various protein preparations, the PS II membranes were washed twice with 100 mM NaCl, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove unbound MSP and then resuspended in 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to a chl concentration of 1 mg/mL. These samples were then solubilized in LiDS and electrophoresed on 15% polyacrylamide gels and Western blotted onto polyvinylidene difluoride (PVDF) membranes, which were blocked as described previously (*17*). Known concentrations of the various modified proteins were simultaneously run on the same polyacrylamide gels as the reconstituted PS II membranes (*18*, *19*). The Western blots were then probed with a polyclonal antibody (see below) that was directed toward the N-terminus of the MSP and the bands were visualized as described previously (*18*). After drying, the Western blots were scanned with a Microtek E6 scanner (300 dpi resolution, 256 gray-scale levels) and the integrated optical density of the MSP bands was determined by the program SigmaGel version 1.0 (Jandel Scientific Software).

The polyclonal antibody used in these studies recognizes the N-terminus of the MSP. A chemically synthesized peptide comprising the first 29 N-terminal residues of the mature MSP (NtMSP: EGGKRLTYDEIQSKTYLEVKGTANQCP) was conjugated to keyhole limpet hemocyanin (*20*). Antiserum was produced and isolated from rabbits after their immunization by standard protocols. To confirm its specificity, the antiserum was tested against NtMSP-coupled keyhole limpet hemocyanin, the NtMSP peptide, and the MSP on dot blots and against the NtMSP peptide, MSP, and PS II membranes on Western blots (data not shown). These experiments confirmed the specificity of the antiserum preparation for the N-terminus of the MSP.

For the identification of GME-modified domains, 1 mg of the modified MSP was brought to 10% TCA. The protein precipitate was collected by centrifugation and then washed twice with cold 100% acetone and dried under vacuum. The protein pellet was then dissolved in 50 μL of 8.0 M urea, 400 mM ammonium bicarbonate, and 4.5 mM DTE and incubated at 50 °C for 15 min. After the sample was cooled to room temperature, 1.6 μL of 4-vinylpyridine was added to block sulfhydryl groups (*21*). The protein sample was then diluted 4-fold and incubated overnight with either *Staphylococcus* V8 protease or TPCK-treated trypsin. Both proteases were sequencing-grade reagents. The samples were then either directly analyzed by MALDI mass spectrometry (see above) or the peptides were first extracted on a reversed-phase (C_{18}) chromatographic material (Ziptips, Millipore Corp.) as per manufacturer's directions prior to mass spectrometry analysis. Masses of the proteolytic fragments were generally reported as average masses; however, in some particularly well resolved spectra, monoisotopic masses were obtained and reported (see Tables 2 and 3).

RESULTS AND DISCUSSION

Characterization of GME−EDC-Modified MSPs. Figure 1A illustrates the relative migration in LiDS−PAGE of the various MSP preparations used in this study. Unmodified MSP migrated with an apparent molecular mass of 33 kDa, the MSP that had been modified in solution with GME exhibited an apparent molecular mass of 28 kDa, and the MSP that had been modified while associated with the PS II membrane exhibited an apparent molecular mass of 30 kDa. These results are similar to those reported by Miura et al. (*10*). Modification of carboxylates on the MSP leads to an increase in its relative mobility during LiDS−PAGE. Additionally, the protein bands of the modified proteins are more diffuse, and consequently exhibit lower staining densities, than the unmodified MSP. Figure 1B,C demonstrates that all three of the MSP preparations used in this study react similarly with the polyclonal antibody reagent anti-NtMSP. Image analysis of the Western blot shown in Figure 1B is illustrated in Figure 1C. Integration of the protein band images indicates that similar (although not identical) optical densities are obtained for the three protein preparations. The modified proteins actually exhibit somewhat enhanced immunological staining vis-à-vis the unmodified protein, particularly at higher protein loads. It should be noted that this difference could lead to a small overestimation of the amount of the modified proteins that are bound to the PS II membranes. This effect was ameliorated by the use of appropriate MSP standards during quantification of the binding experiments (see above; *17*, *18*). Since all three MSP preparations react with this reagent, it can be used to detect the MSP in rebinding experiments shown below. Apparently, none of the carboxylates that are modified in the N-terminal portion of the MSP (see below) are involved in the formation of major antigenic determinants for anti-NtMSP.

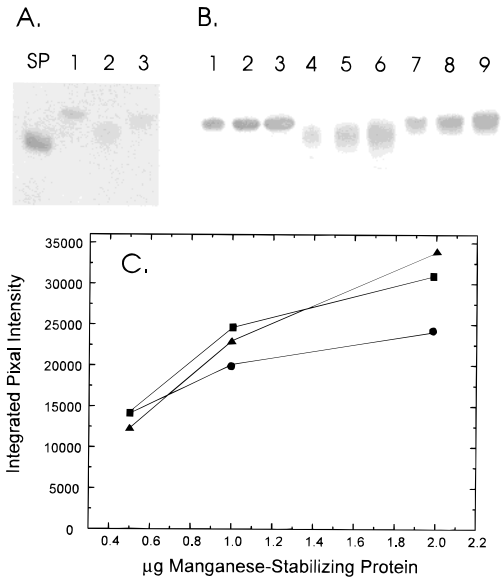

FIGURE 1: Electrophoretic and Western blot analysis of GME−EDC-modified and control MSP. (A) Coomassie blue stain of LiDS−PAGE gel. SP, ovalbumin, 24 kDa; lane 1, unmodified MSP; lane 2, MSP modified in solution; lane 3, MSP modified while associated with PS II. (B) Western blot analysis of MSPs detected with the anti-NtMSP polyclonal antibody. Lanes 1−3 contained 0.5, 1.0, and 2.0 μg, respectively, of unmodified MSP; lanes 4−6 contained 0.5, 1.0, and 2.0 μg, respectively, of MSP that had been modified in solution; and lanes 7−9 contained 0.5, 1.0, and 2.0 μg, respectively, of MSP that had been modified while associated with PS II. (C) Image analysis of the Western blot shown in panel B. The integrated band intensities of the immunoreactive bands were plotted versus the protein concentration. (●) Unmodified MSP; (■) MSP that had been modified in solution; (▲) MSP that had been modified while associated with PS II.

Table 1: MALDI Mass Spectrometry of GME−EDC-Modified Manganese- Stabilizing Protein

protein	mass[a] (Da)	Δmass[b] (Da)	no. of GME modifications
unmodified MSP	26 523.4 (σ = 11.6)	NA[d]	NA[d]
MSP modified on PS II membranes	26 636.0 (σ = 9.5)	102.3	1−2
MSP modified in solution	26 828.3 (σ = 25.3)	294.6	4

[a] Mass determined by MALDI mass spectrometry. Unmodified MSP has a mass calculated to be 26 533.7 Da on the basis of its derived amino acid sequence (*22*). σ = 1 standard deviation, n = 3. [b] Δmass = observed mass − 26 533.7 Da. [c] Each GME modification adds 72.09 Da. [d] Not applicable.

MALDI mass spectrometry was used to determine the number of carboxylates that were modified by GME−EDC treatment (Table 1). The unmodified MSP exhibits a mass of 26 523.4 Da, which is in excellent agreement with the mass calculated from its derived amino acid sequence (26 533.66 Da) (*22*) with a mass error of 0.04%. Modification with GME−EDC either in solution or when the MSP is associated with the PS II membranes leads to an increase in

the molecular mass of the modified proteins. These mass increases are consistent with the modification of 1−2 carboxylates on the protein modified in association with the membranes and 4 carboxylates on the protein that was modified in solution.

Miura et al. (*10*) also modified the MSP under both labeling conditions. They found that modification with GME−EDC leads to a significant shift in the pI of the modified protein (5.2 for unmodified protein, 8.4 for the protein modified on the membranes, and 8.6 for the protein modified in solution). They calculated that this p*I* shift was due to the modification of about eight carboxylates under either labeling condition [by use of the p*I*/M_r program (*23*)]. Since our labeling protocols were very similar to those used in ref *10*, we cannot account for this discrepancy. It should be noted, however, that a similar program, Protein Tools (Perspective Biosoftware), yields a calculated p*I* for the MSP of 4.96. This is significantly lower than the observed p*I* for this protein of 5.2 (*24*). Using the p*I* data from Miura et al. (*10*), this program also calculates a modification of 8 and 9 carboxylates for the MSP modified on the membranes and in solution, respectively. If, however, the p*I* calculation is normalized to the actual p*I* of the MSP (i.e., p*I* = 5.2) the program calculates the modification of 5 and 6 carboxylates for the MSP modified on the membranes and in solution, respectively. These values are closer, although not identical, to the values that we obtained by direct mass measurement using MALDI-mass spectrometry. We conclude that the p*I* analysis performed in ref *10* overestimates the number of GME modifications that are present in the modified MSPs.

It should be noted that EDC can also modify tyrosyl (*25*) and cysteinyl residues (*26*) but at a 20-fold lower efficiency than carboxylate-containing residues. Modification of tyrosyl residues with EDC involves the formation of an acid-stable *o*-arylisourea and is not dependent on the presence of GME. The adduct formed upon reaction of EDC with free sulfhydryls is also stable and also does not require the presence of GME. Under the conditions employed in our experiments, the EDC concentration is kept very low (i.e., 2 mM vs 100 mM) in order to minimize these alternative modifications. Additionally, the cysteinyl residues present in the MSP are associated, forming a disulfide bond (*27*). Disulfides are not susceptible to modification by EDC. EDC can also catalyze the formation of inter- and intramolecular protein cross-links between carboxyl-containing amino acids and lysyl residues (*28*). In our study, this reaction is suppressed by the presence of relatively high concentrations of GME. Nevertheless, a small amount of intramolecularly cross-linked MSP can be detected immunologically (data not shown). We estimate that this constitutes less than 5% of the total modified MSP used in our experiments.

Binding of Modified MSPs to PS II. The ability of the GME−EDC-modified proteins to bind to PS II membranes and to reconstitute oxygen evolution is shown in Figures 2 and 3. In Figure 2, MSP-depleted PS II membranes that contained 4 manganese per reaction center were incubated with various amounts of either unmodified or GME−EDC-modified MSP. The membranes were washed extensively and the amount of bound MSP was analyzed by LiDS−PAGE, followed by Western blotting, probing with an MSP-specific polyclonal antibody, and image analysis. The unmodified MSP bound freely to PS II membranes, with

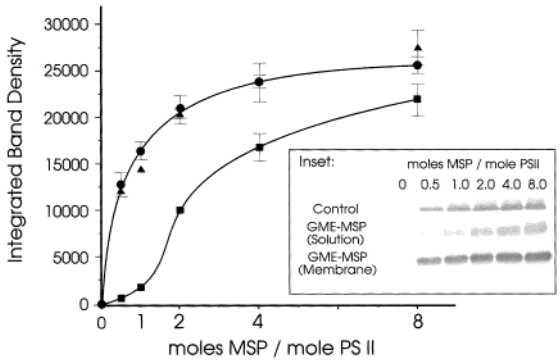

FIGURE 2: Rebinding of GME−EDC-modified and control MSPs to $CaCl_2$-washed PS II. (●) Unmodified MSP; (■) MSP that had been modified in solution; (▲) MSP that had been modified while associated with PS II. Error bars are ±1.0 standard error. Please note that some error bars are smaller than the data point symbols. Inset: representative immunostained Western blots of PS II membranes that had been reconstituted with control MSP, MSP that had been modified in solution, and MSP that had been modified while associated with the PS II membrane.

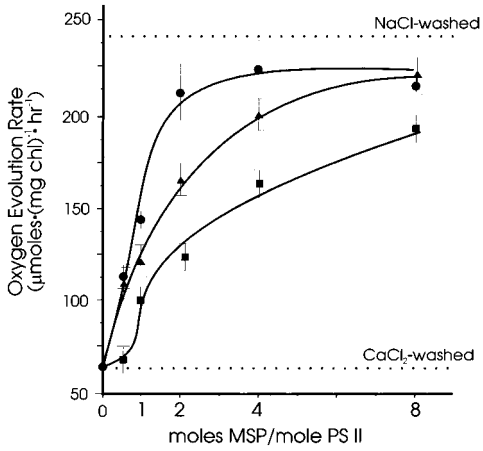

FIGURE 3: Reconstitution of oxygen evolution activity by modified and control MSPs. The activities of NaCl-washed and $CaCl_2$-washed PS II membranes are indicated by dotted lines. The activity of unwashed PS II membranes was 510 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}. (●) Unmodified MSP; (■) MSP that had been modified in solution; (▲) MSP that had been modified while associated with PS II.

saturation being reached at about 2 mol of added MSP/mol of PS II. The binding of MSP that had been modified with GME−EDC while associated with the PS II membranes was indistinguishable from that observed with the unmodified protein. Reconstitution with MSP that had been modified in solution, however, yielded a radically different binding curve. Binding of this protein was markedly sigmoidal and did not exhibit full saturation even at 8 mol of added protein/mol of PS II. This result indicates that modification of the additional carboxylates exposed when MSP was labeled in solution dramatically alters the ability of the modified protein to bind to PS II.

Analysis of this binding data allows an estimation of the dissociation constants for the MSP that had been modified in solution. Earlier we had reported that unmodified MSP exhibits dissociation constants of 2.4 and 1.5 nM, respectively, for the two copies of the MSP associated with the PS II membranes (*19*). The MSP that was modified in solution exhibits dissociation constants of about 1800 and 80 nM.

This represents a dramatic decrease in the affinity of this modified MSP for PS II. It should be noted that the dissociation constants reported here for the modified proteins are only approximate. These calculations were based on far fewer data points (6 vs 20) than in the previous study.

In Figure 3, the ability of the modified MSPs to reconstitute oxygen evolution was examined. The unmodified protein reconstituted oxygen evolution, with the oxygen evolution rate saturating at about 2 mol of MSP/mol of PS II. The MSP modified in solution exhibited sigmoidal oxygen evolution reconstitution that never attained saturation, while the MSP that was modified on the PS II membranes exhibited an intermediate ability to reconstitute oxygen evolution. This experiment indicates that while binding of the MSP modified in solution was compromised, the modified protein was nevertheless competent in functionally reconstituting oxygen evolution (>85% of control at a 8:1 MSP/PS II ratio).

Comparison of the MSP binding curves illustrated in Figure 2 with the oxygen evolution reconstitution experiments shown in Figure 3 is interesting. Two major differences are apparent. First, while modification of the MSP in association with the PS II membrane yields a protein that exhibits apparently completely normal binding, this modified protein is not capable of normally reconstituting oxygen evolution at low MSP/PS II ratios. Second, examination of the binding curves and oxygen evolution reconstitution curves for the MSP that had been modified in solution reveals an apparent discrepancy. A smaller amount of the MSP appears to be bound (Figure 2) than can account for the amount of reconstitution observed (Figure 3), particularly at 1.0 mol of MSP/mol of PS II reaction center. This is due to the fact that, in the MSP binding experiment, the membranes were extensively washed following binding of the MSP. Because of the lowered binding affinity of the modified protein, a substantial amount would be expected to be released during these washes. In the oxygen evolution reconstitution experiment, the membranes were not washed following MSP addition. The modified MSP, which exhibits low affinity for the PS II membranes, was not removed and can contribute to the reconstitution of oxygen evolution activity.

These results differ significantly from those described in ref *10*. They reported that the modification of carboxylates on the MSP modified in solution had no effect on the binding of this component to PS II. This erroneous conclusion was reached because they carried out their binding studies at a single, high molar ratio of MSP to PS II (about 6:1 MSP/PS II) and did not examine a binding curve. At this high ratio only a small difference in binding would have been observed. We do, however, confirm their observation that MSP that has been modified in solution can reconstitute oxygen evolution activity at high MSP/PS II ratios. They observed 100% reactivation of oxygen evolution, while we observed an 85% reactivation at a MSP/PS II ratio of 8:1.

It should be noted that the observation of sigmoidal binding and oxygen reactivation curves supports the hypothesis that two copies of the MSP are associated with PS II. A variety of other lines of evidence also support this hypothesis. Yamamoto et al. (*29*) purified the MSP from spinach PS II membrane preparations using a high-efficiency butanol extraction procedure. They isolated 1.75 mol of the MSP extrinsic protein/mol of PS II reaction center. Since protein purification is never 100% efficient, this result underestimates the actual amount of the MSP that is associated with PS II membranes. In our laboratory (*18*), we directly measured the amounts of the MSP associated with both PS II membranes and oxygen-evolving PS II core preparations from spinach. Our results indicated that PS II membranes and the oxygen-evolving core complex contain 2.0 mol of the MSP/mol of PS II reaction center. We also examined the rebinding of the MSP to PS II membranes that contained 0, 2, or 4 manganese/PS II reaction center (*18*). In all cases a stoichiometry of 2 mol of MSP/mol of PS II was observed and the binding of the MSP exhibited positive cooporativity (Hill coefficients of 1.6−2.1). Other rebinding experiments have supported these findings (*30*). Further evidence for the presence of two copies of the MSP was provided by the differential displacement of mutant MSP by wild-type protein (*31*). Wild-type MSP could displace precisely half of the MSP that contained a mutation at position ^{235}V from reconstituted PS II membranes. This indicated the presence of two binding sites for the MSP. A final line of evidence supporting a stoichiometry of 2 for the MSP also arises from mutant studies. C. F. Yocum (personal communication) pointed out that a number of independent MSPs exhibit binding curves that can only be explained by the presence of multiple binding sites. These mutant MSPs exhibit lower apparent binding affinity for PS II and many also exhibit obviously sigmoidal binding curves. For instance, the 33-His mutant, which bears a His tag at the C-terminus of the spinach MSP, exhibits such a curve (*32*). These results indicate the presence of at least two cooperative binding sites. If there were only a single binding site for the 33 kDa protein, a mutant with a lowered binding affinity would exhibit a normal rectangular hyperbolic binding curve with no sigmoidicity.

Our results demonstrate that at least two populations of carboxylates appear to be present on the MSP. The first population can be modified when the MSP is associated with PS II. This population appears to consist of 1−2 carboxylate groups, the modification of which leads to no alteration in the binding of the protein to PS II although the functional characteristics of the protein are somewhat altered. The second population of carboxylates is modified only when the MSP is labeled in solution. This population appears to consist of two additional modifiable carboxylates. The modification of these additional groups leads to a dramatic decrease in the ability of the MSP to associate with the photosystem. Once associated with the photosystem, however, the modified MSP is able to functionally reconstitute oxygen evolution.

Mapping the Sites of GME−EDC Modification. In an effort to understand which domains on the MSP are responsible for its association with PS II, we have identified the regions of the MSP that are differentially labeled with GME−EDC. Table 2 lists the peptides obtained by digestion of the MSP that had been labeled on the membrane, while Table 3 lists the tryptic and V8 protease peptides obtained from digestion of the MSP which had been labeled in solution.

In these studies, two proteases (trypsin or *Staphylococcus* V8 protease) and two matrixes (cyano-4-hydroxycinnamic acid or sinapinic acid) were employed. The combination of different proteases, which differentially cleave the MSP, and

Table 2: Assignments for Peptides Produced from Either Trypsin or *Staphylococcus* V8 Protease Digestion of the MSP That Was Modified on the PS II Membrane

observed mass[a] (Da)	predicted mass[b] (Da)	Δmass	matrix	peptide assignment
		Tryptic Peptides		
753.33	752.88	0.45	ca[c]	^{15}T−^{20}K
851.14	850.94	0.20	ca	152G−^{159}K
1254.10	1253.39	0.71	ca	5R−^{14}K
1389.63	1388.71	0.92	ca	^{50}F−^{60}K + 1 VP[d]
1626.02	1624.78	1.24	ca	1E−^{14}K
1680.82	1681.88	−1.06	ca	^{67}N−^{80}R + 1 GME[e]
1677.99	1677.87	0.12	sa[f]	^{234}D−^{247}Q
1695.13	1693.80	1.33	ca	^{61}A−^{76}R
1777.50	1776.90	0.60	ca	^{160}G−^{178}R
1831.70	1831.06	0.64	ca	^{6}L−^{20}K
2164.98	2164.28	0.70	ca	^{179}G−^{196} K+ 2 GME
2267.67	2267.54	0.13	sa	^{61}A−^{80}R+ 1 GME
2285.13	2285.55	−0.42	sa	^{131}Q−^{151}R
2476.01	2474.75	1.26	ca	^{21}G−^{44}K + 1 VP
2534.03	2533.02	1.01	ca	^{45}G−^{66}K + 1 GME
2940.30	2938.28	2.02	ca	^{21}G−^{48}K + 1 VP
2980.70	2978.11	2.59	ca	^{162}G−^{190}K
3068.20	3066.46	1.74	ca	^{21}G−^{49}K + 1 VP
3115.62	3117.48	−1.86	sa	^{131}Q−^{159}K
3241.20	3241.70	−0.50	sa	^{102}F−^{130}K
3305.10	3303.84	1.26	ca	^{123}V−^{151}R + 1 GME
3464.30	3464.84	−0.54	sa	^{197}G−^{230}K
3567.40	3567.01	0.39	ca	^{15}T−^{48}K
3589.96	3588.18	1.78	ca	^{49}K−^{80}R
3624.12	3622.15	1.97	ca	^{45}Y−^{76}K+ 1 GME
3780.86	3780.31	0.55	ca	^{21}G−^{56}K
3788.41	3789.26	−0.85	sa	^{197}G−^{233}K
3837.26	3839.35	−2.09	ca	^{15}T−^{49}K + 2 GME
4576.48	4575.22	1.51	sa	^{81}L−^{122}R
4643.45	4645.19	−1.74	sa	^{6}L−^{48}K
4725.04	4724.45	0.59	ca	^{15}T−^{56}K + 2 VP
4819.85*	4819.40	0.45	ca	^{6}L−^{48}K + 1 GME + 1 VP
		V8 Peptides		
1870.66	1871.00	−0.34	ca	^{88}I−^{104}E
2030.12	2031.44	−0.05	ca	^{122}R−^{139}E
2263.45	2263.50	−0.05	ca	^{188}N−^{209}E
2276.88	2275.51	1.37	ca	^{1}E−^{18}E + 2 GME
2511.76	2512.84	−1.08	ca	^{11}I−^{32}E + 2 GME
2785.48	2785.20	0.28	ca	^{213}V−^{238}E
2831.34	2831.17	0.17	ca	^{63}G−^{87}E
2983.60	2985.24	−1.64	ca	^{94}V−^{121}E + 1 GME
3372.43	3371.96	0.47	ca	^{33}G−^{62}E + 1 VP
3584.52	3585.89	−1.37	ca	^{88}I−^{121}E
3746.73	3746.33	0.40	ca	^{105}K−^{139}E
4525.76	4524.81	0.95	ca	^{140}S−^{183}E
5024.58	5023.39	1.19	ca	^{140}S−^{187}E
5599.47	5598.32	1.15	ca	^{88}I−^{139}E

[a] An asterisk indicates monoisotopic mass; all others are average masses. [b] Mass predicted from analysis of the *psbO* gene (*22*). In cases of modified peptides, the masses of the modification have been added to the predicted peptide mass. [c] Cyano-4-hydroxycinnamic acid. [d] Each VP modification adds 105.14 Da. [e] Each GME modification adds 72.09 Da. [f] Sinapinic acid.

Table 3: Assignments for Peptides Produced from Either Trypsin or *Staphylococcus* V8 Protease Digestion of the MSP That Was Modified in Solution

observed mass[a] (Da)	predicted mass[b]	Δmass	matrix	peptide assignment
		Tryptic Peptides		
1097.53*	1096.54	0.99	ca[c]	^{6}L−^{14}K
1136.20	1136.27	−0.07	ca	^{152}G−^{161}R + 1 GME[d]
1635.72*	1634.79	0.93	ca	^{162}G−^{178}R + 1 GME
1778.12	1776.90	1.22	ca	^{160}G−^{178}R
2267.20*	2266.13	1.07	ca	^{61}A−^{80}R + 1 GME
2284.25*	2285.55	0.10	ca	^{131}Q−^{151}R
2533.94	2533.02	0.92	ca	^{45}G−^{66}K+ 1 GME
2708.47	2708.12	0.35	ca	^{106}D−^{130}K
2936.47*	2938.28	0.05	ca	^{21}G−^{48}K + 1 VP[e]
3067.84	3066.46	1.38	ca	^{21}G−^{49}K + 1 VP
3115.53*	3118.44	−0.04	sa[f]	^{131}Q−^{159}K
3567.42	3567.01	0.41	ca	^{15}T−^{48}K
3624.16	3622.15	2.01	ca	^{45}Y−^{76}K + 1 GME
3780.17	3780.31	−0.14	ca	^{21}G−^{56}K
3788.45	3789.26	−0.81	sa	^{197}G−^{233}K
3837.43	3839.35	−1.92	ca	^{15}T−^{49}K + 2 GME
3850.25	3850.08	0.17	ca	^{160}G−^{196}K + 1 GME
		V8 Peptides		
1093.76	1094.20	−0.44	sa	^{239}G−^{246}E
1168.61	1168.24	0.37	ca	^{1}E−^{10}E
1510.75	1510.70	0.05	ca	^{19}V−^{32}E + VP
1628.22	1628.72	−0.50	ca	^{90}G−^{104}E
1735.14	1733.91	1.23	ca	^{105}K−^{121}E
1871.40	1871.00	0.40	ca	^{88}I−^{104}E
2000.45	2002.00	−1.77	ca	^{2}G−^{18}E
2029.99*	2031.44	−0.18	sa	^{122}R−^{139}E
2189.33	2189.42	−0.09	ca	^{1}E−^{18}E + 1 GME
2264.63	2263.5	1.13	ca	^{188}N−^{209}E
2786.21	2785.20	1.01	ca	^{213}V−^{238}E
2832.42	2831.17	1.25	ca	^{63}G−^{87}E
3074.16	3073.44	0.72	ca	^{63}G−^{89}E
3584.14	3585.89	−1.75	sa	^{88}I−^{121}E
3729.42	3730.07	−0.65	sa	^{88}I−^{121}E + 2 GME
3743.25*	3746.33	−0.78	ca	^{105}K−^{139}E
3801.42	3798.49	−2.93	sa	^{213}V−^{246}E + 1 GME
4221.7	4219.75	1.95	ca	^{210}T−^{247}Q + 1 GME
4593.47	4596.90	−3.43	sa	^{140}S−^{183}E + 1 GME
4751.88	4755.24	−3.36	sa	^{63}G−^{104}E + 1 GME
5020.44	5023.39	−2.95	sa	^{140}S−^{187}E
5595.90*	5594.89	1.01	ca	^{88}I−^{139}E

[a] An asterisk indicates monoisotopic mass; all others are average masses. [b] Mass predicted from analysis of the *psbO* gene (*22*). In cases of modified peptides, the masses of the modification have been added to the predicted peptide mass. [c] Cyano-4-hydroxycinnamic acid. [d] Each GME modification adds 72.09 Da. [e] Each VP modification adds 105.14 Da. [f] Sinapinic acid.

different matrixes, which differentially mobilize the proteolytic peptides into the vapor phase, greatly increases the number of peptides that can be analyzed in these experiments. Increasing the number of peptides that can be analyzed increases the probability that a representative and more complete sample of proteolytic fragments will be obtained for analysis. Indeed, in these studies, near 100% coverage for both MSP modified on the PS II membrane and MSP modified in solution was obtained.

Overnight digestion of the modified MSPs with either trypsin or *Staphylococcus* V8 protease yielded a mixture of completely and partially digested proteolytic fragments. Such peptide mixtures can be analyzed with high mass accuracy by MALDI mass spectrometry (*10*, *17*). In this paper, peptide mass assignments were made within a 0.2% mass error envelope (i.e., ±0.1%) surrounding the theoretical peptide mass, which was determined from the known amino acid sequence of the MSP (*22*). The program GPMAW version 3.0 was used to facilitate the peptide assignments.

Two protein modifications were introduced into the MSP during the course of these experiments. First, the protein was labeled with GME in an EDC-facilitated reaction, either on NaCl-washed PS II membranes or free in solution. This modification resulted in the addition of 72.09 Da for each modified carboxylate group. Second, prior to proteolytic

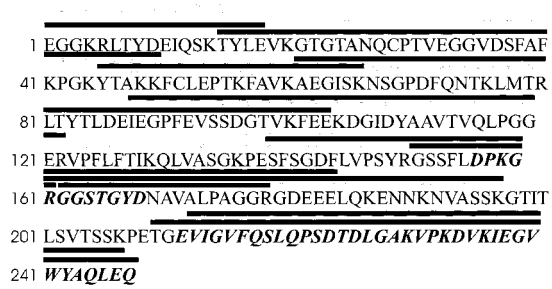

FIGURE 4: Location of representative GME−EDC-modified tryptic and *Staphylococcus* V8 protease peptides. The amino acid sequence of spinach MSP (*22*) is shown. GME-modified peptides that were labeled in solution are indicated by black lines above, while GME-modified peptides that were labeled on the membrane are indicated by gray lines above. The domains that were modified *only* in solution and that contain the carboxylates responsible for the altered binding affinity of the MSP are shown in boldface italics type.

digestion, the modified MSP was reduced with DTE and treated with 4-vinylpyridine. This blocked the free sulfhydryl groups on the protein, which greatly enhances the effectiveness of the protease treatment (*33*), and added 105.14 Da per modified cysteine.

For the protein that was modified on the PS II membrane, eight tryptic peptides and three *Staphylococcus* V8 protease peptides were modified with GME−EDC. Since the MSP that had been modified on the membrane contained two modified carboxylates (Table 3), it is apparent that different carboxylates were modified on different individual MSP molecules. This is similar to the results obtained by modification of the MSP with NHS-biotin (*17*), *N*-succinimido propionate, and 2,4,6-trinitrobenzene sulfonic acid (*10*). Additionally, in some instances, both modified and unmodified versions of the same peptide were identified (for instance, ^{6}L−^{48}K). This has also been observed for the other labeling systems (*10*, 17).

These results indicate that the domains ^{1}E−^{144}D and ^{180}D−^{187}E can be labeled when the MSP is associated with the PS II membrane. A word of caution is necessary in the interpretation of these results. It is possible that the removal of the 24 and 17 kDa components by 1.0 M NaCl treatment could induce conformational changes in the bound MSP. This could lead to alterations in the labeling pattern of the membrane-bound protein. No such conformational changes, however, have ever been identified.

For the MSP that was modified in solution, seven tryptic and six *Staphylococcus* V8 protease peptides were modified with GME−EDC. These results indicate that the domains ^{1}E−^{121}E, ^{144}D−^{187}E, and ^{212}E−^{247}Q (C-terminus) can be modified when the MSP is reacted with GME−EDC in solution.

The results of these experiments are summarized in Figure 4. The two domains ^{157}D−^{168}D and ^{212}E−^{247}Q are labeled only when the MSP is modified in solution. Two hypothetical mechanisms could explain the differential modification observed for these regions. First, the MSP could undergo significant conformational changes upon release from PS II that lead to the exposure of carboxylate groups normally buried and inaccessible in the interior of the protein. A second hypothesis is that the MSP undergoes no large conformational change upon release from PS II and that the carboxylate groups are normally exposed on the surface of the protein. These carboxylates are shielded, however, from the modification reagents by interaction with intrinsic PS II components. At this time it is difficult to differentiate between these two hypotheses and it should be noted that these hypotheses are not necessarily mutually exclusive.

Earlier investigations have also identified these domains as possibly being involved in MSP−PS II interactions. A site-directed mutation at the conserved residue ^{209}E in *Synechocystis* MSP (which corresponds to residue ^{212}E in spinach) led to a 30−40% loss in oxygen evolution activity. Additionally, this mutant (E209Q) exhibits weaker binding to PS II (R. Burnap, unpublished observations). These phenotypes are consistent with those expected upon disruption of the MSP−PS II interaction. Betts et al. (*31*, *34*, *35*) have also examined the effects of mutations near the C-terminus of the MSP. Alteration of ^{235}V to ^{235}A in spinach MSP yielded a protein that could not bind efficiently to PS II membranes at 4 °C but was competent at 22 °C (*34*). Additionally, this mutant protein could bind strongly to only one of two MSP binding sites (*31*). C-Terminal truncations of spinach MSP also lead to altered binding and oxygen-evolution reconstitution characteristics (*35*). Sequential removal of the three C-terminal amino acid residues leads to progressively more severe phenotypes. Motoki et al. (*36*) demonstrated that proteolytic cleavage of the MSP between ^{156}F and ^{163}G led to a loss in the ability of the cleaved MSP to rebind to PS II. Finally, we had earlier identified the domain ^{149}S−^{171}V as a loop lying between two β-strand domains of the MSP (*37*). This loop appears to interact with the intrinsic PS II components.

It is clear that modification of carboxylates in the domains ^{157}D−^{168}D and/or ^{212}E−^{247}Q has a profound effect on the ability of the modified MSP to bind to PS II. A number of mechanisms could account for our observations. First, it is possible that the modified carboxylates are directly involved in the formation of charge-pair interactions with the intrinsic components of the photosystem. In earlier work, we identified positively charged residues on CP 47 that appear to form a binding domain for the MSP. Conversion of the ^{384}R and ^{385}R residues in CP 47 to glutamates (yielding the mutant RR384385EE) essentially abolishes the binding of MSP to PS II and yields a phenotype very similar to that of Δ*psbO* (*38*). Alteration of these arginyl residues, either individually or in tandem, to glycyl residues leads to phenotypes that are less extreme than observed for the RR384385EE mutant (*38*, *39*). It is tempting to hypothesize that carboxylates located in the domains ^{157}D−^{168}D and/or ^{212}E−^{247}Q may participate in charge-pair interactions with ^{384}R and ^{385}R of CP 47. However, no direct evidence is available to support this hypothesis.

A second possibility is that the conjugation of glycine methyl ester to the carboxylates sterically interferes with the association of MSP with PS II. The presence of the glycine moiety may prevent the formation of protein−protein interactions that are required for effective binding of MSP to PS II. Finally, since it is apparent that conformational changes in MSP may occur during its association with PS II (*40*), it is possible that these or other conformational changes may be *required* for efficient binding. Modification of carboxylates on the MSP may prevent necessary conformational changes from occurring efficiently, thus lowering the affinity of the MSP for PS II. Betts et al. (*31*) have

hypothesized that conformational changes in the MSP that take place upon its binding to PS II produces the observed high-affinity binding of this component. The proposal that the MSP possesses a "natively unfolded" solution structure (*41*) may support this hypothesis. It is unclear, at this time, which of these hypotheses best explains the dramatic effects on the binding to PS II of the MSP that has been modified in solution. The experimental differentiation of these hypotheses constitutes an area of continuing investigation.

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