Carboxylate Groups on the Manganese-Stabilizing Protein Are Required for Efficient Binding of the 24 kDa Extrinsic Protein to Photosystem II†

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ABSTRACT: The effects of the modification of carboxylate groups on the manganese-stabilizing protein on the binding of the 24 kDa extrinsic protein to Photosystem II were investigated. Carboxylate groups on the manganese-stabilizing protein were modified with glycine methyl ester in a reaction facilitated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The manganese-stabilizing protein which was modified while associated with NaCl-washed membranes could bind to calcium chloride-washed PS II membranes and reconstitute oxygen evolution in a manner similar to that observed for unmodified manganese-stabilizing protein (Frankel, L.K, Cruz, J. C. and Bricker, T. M. (1999) *Biochemistry 38,* 14271−14278). However, PS II membranes reconstituted with this modified protein were defective in their ability to bind the extrinsic 24 kDa protein of Photosystem II. Mapping of the sites of modification was carried out by trypsin and *Staphylococcus* V8 protease digestion of the modified protein and analysis by MALDI mass spectrometry. These studies indicated that the domains ^{1}E−^{71}D, ^{97}D−^{144}D, and ^{180}D−^{187}E are labeled when the manganese-stabilizing protein is bound to NaCl-washed Photosystem II membranes. We hypothesize that modified carboxylates, possibly residues ^{1}E, ^{32}E, ^{139}E, and/or ^{187}E, in these domains are responsible for the altered binding affinity of the 24 kDa protein observed.

In higher plants and cyanobacteria, at least six intrinsic proteins appear to be required for oxygen evolution by Photosystem II (PS II) (*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 16 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. The 24 and 16 kDa proteins appear to modulate the calcium and chloride requirements for efficient oxygen evolution. These three extrinsic components interact with intrinsic membrane proteins and possibly with each other to yield fully functional oxygen-evolving complexes.

The binding determinants for the MSP to PS II have been extensively studied (for reviews, see refs *6*−*8*). Arginyl residues at positions 384 and 385 on the CP47 protein appear to form a major binding domain for the MSP in cyanobacteria (*9*−*11*). Positively charged residues at these positions are conserved in all of the CP47 sequences acquired to date. The specific amino acid residues present on the MSP which are required for its interaction with PS II have not been rigorously identified; however, the importance of residues located in the N-terminus of the MSP in this interaction is now clear (*12*−*15*).

Much less is known about the binding domains for the 24 and 16 kDa proteins within the photosystem. In reconstitution experiments with PS II membranes preparations, the MSP appeared to be required for the binding of the 24 kDa protein, and the 24 kDa protein appeared to be required for the binding of the 16 kDa protein (*16*, *17*). However, differential extraction experiments using a butanol−water phase separation system (*18*) or Hg^{2+} (*19*) indicated that the MSP could be removed from PS II preparations without the concomitant extraction of the 24 and 16 kDa proteins. It is possible that the MSP is required for binding of the 24 kDa component but that upon binding, conformational changes occur within the intrinsic components of PS II which allow the selective removal of the MSP and retention of the 24 and 16 kDa proteins. In any event, the MSP, 24, and 16 kDa proteins must be in close proximity, since cross-linking experiments

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[1] Abbreviations: Chl, chlorophyll; DTE, dithioerythritol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; 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.

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performed with homobifunctional cross-linkers (6−11 Å span) indicated that the MSP is within 11 Å of the 24 kDa protein and that the 24 kDa protein is within 11 Å of the 16 kDa component. (*20*). Finally, in cross-reconstitution experiments, $CaCl_2$-washed spinach PS II membranes which had been reconstituted with either cyanobacterial or red algal MSP could only partially rebind spinach 24 kDa protein and could not bind spinach 16 kDa protein. This indicates that there are structural determinants present on the spinach MSP which are required for the efficient binding of the 24 and 16 kDa proteins and which are absent in the cyanobacterial and red algal proteins (*21*).

In this study, we have examined the role the carboxylates on the MSP play in the binding of the 24 kDa component to PS II. We have found that the modification of carboxylates on the MSP modulate the binding of the 24 kDa protein to the photosystem. We have also mapped the regions on the MSP which contain the modified carboxylate groups.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as previously described (*22*). Chl concentration was measured by the method of Arnon (*23*). Oxygen-evolving PS II membranes were prepared by the method of Berthold et al. (*24*) as modified by Ghanotakis and Babcock (*25*). Typical preparations exhibited a chl *a*/*b* ratio of 1.9−2.0 and an oxygen evolution rate of about 500 μmol O_2 (mg chl)$^{-1}$ h^{-1}. The extrinsic 24 and 16 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 on the NaCl-washed PS II membranes was performed as described previously (*26*). This reaction, which is facilitated by the presence of EDC, leads to the modification of aspartyl and glutamyl residues (*27*). Additionally, the C-terminus of the MSP could 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 (*28*). The MSP present on 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 5 mM MES−NaOH, pH 6.0, filtered through a 0.3 μm filter to remove residual membrane fragments, and concentrated by centrifugal ultrafiltration.

Reconstitution experiments were performed using unmodified MSP and MSP which had been modified while associated with the PS II membranes but in the absence of the 24 and 16 kDa proteins (modified MSP). 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 the different MSP preparations at a MSP/PS II ratio of 5:1. We have shown previously that this high MSP to PS II ratio saturates all of the MSP-binding sites on PS II (*26*) regardless of the extent of modification by GME. After washing twice with 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove unbound MSP, the PS II membranes were resuspended at 50 μg/mL chl in the same buffer and incubated with different concentrations of unmodified 24 kDa protein which had been purified essentially as described previously (*29*). The 24 kDa was quantified using an extinction coefficient of 22 mM^{-1}cm^{-1} at 277 nm (*30*).

For the direct analysis of protein binding after incubation with the various protein preparations, the PS II membranes were washed three times with 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM MES−NaOH, pH 6.0, to remove unbound 24 kDa protein 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 0.5 mg/mL. These samples were then solubilized in LiDS and electrophoresed on 10−20% polyacrylamide gradient gels and Western blotted, and the PVDF membranes were blocked as described previously (*31*). The Western blots were then probed with a polyclonal antibody against the 24 kDa protein (kindly provided by Dr. Ralph Henry), and the bands were visualized as described previously (*32*). After drying, the Western blots were scanned (300 dpi resolution, 256 gray-scale levels), and the integrated optical densities of the 24 kDa bands were determined using the program SigmaGel ver. 1.0 (Jandel Scientific Software).

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 8.0 M urea, 400 mM ammonium bicarbonate, and 4.5 mM DTE and incubated at 50 °C for 15 min. After cooling to room temperature, 1.6 μL of 4-vinylpyridine was added to block sulfhydryl groups (*33*). 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 below), or the peptides were first extracted on a reversed-phase (C_{18}) chromatographic material (Ziptips^{tm}, 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 (Table 1).

MALDI mass spectrometry was performed on the digested 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

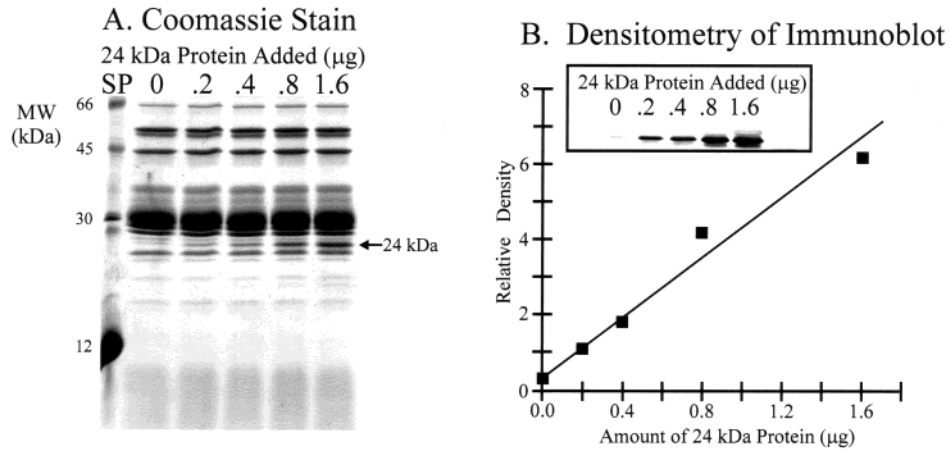

FIGURE 1: Electrophoretic and Western blot analysis of the 24 kDa protein used in the reconstitution studies. (A) Coomassie blue stain of LiDS−PAGE gel. The amounts of the 24 kDa protein added to $CaCl_2$-washed PS II membranes (10 μg or chl) are shown above. The membranes were then immediately solubilized and subjected to electrophoresis. The location of the 24 kDa protein is indicated to the right. Molecular weights of standard proteins are shown to the left. (B) Densitometry of immunoblot. A duplicate of the gel shown in panel A was Western blotted, blocked, and probed with an anti-24 kDa protein polyclonal antibody. After chromogenic development, the blot (shown in inset) was scanned as described in the Materials and Methods section. A plot of relative density vs amount of 24 kDa protein added indicated that the antibody response was linear throughout the range of added 24 kDa protein used in the subsequent reconstitution studies.

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). Mass spectra for the proteolytic fragments were calibrated using external standardization. The MALDI mass spectra were analyzed using the GPMAW Program ver. 3.0 (Lighthouse Data, Denmark).

RESULTS AND DISCUSSION

The characteristics of the MSP preparations used in this study have been described previously (*26*). In short, unmodified MSP exhibited a molecular mass of 26 523.4, while the MSP which had been modified on NaCl-washed PS II membranes exhibited a mass of 26 636.0, indicating that one to two carboxylates had been modified on this protein. Both of these proteins bound identically to MSP-depleted PS II membranes, with the binding saturating at about 2 mol MSP per mol PS II. Both of these proteins were also capable of essentially fully reconstituting oxygen evolution at an MSP/PS II ratio of 5:1.

Figure 1 illustrates a typical control experiment in which the 24 kDa protein was added to PS II membranes which had been washed with $CaCl_2$ to remove the extrinsic proteins. After the addition of various amounts of the 24 kDa protein, the samples were immediately solubilized and subjected to electrophoresis. After electrophoresis the separated proteins were either stained with Coomassie blue (Panel A) or Western blotted and probed with an antibody against the 24 kDa protein, chromogenically developed, and digitized. This experiment demonstrates that, in the range of 24 kDa protein used in the subsequent rebinding studies, the antibody yields a linear relationship between density of the chromogenic bands and the amount of 24 kDa protein added to the PS II membranes.

Figure 2 illustrates the results of studies in which the 24 kDa protein was rebound onto PS II membranes which had been reconstituted with either saturating amounts of control

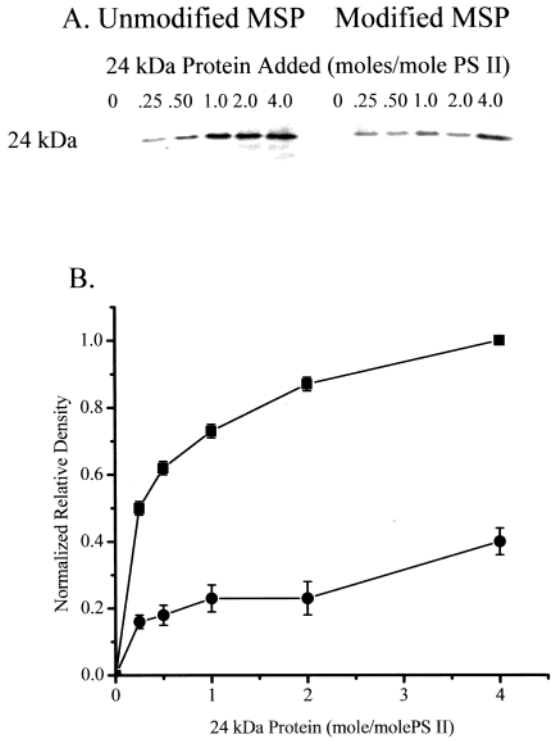

FIGURE 2: The binding of the 24 kDa protein to PS II membranes which had been reconstituted with either unmodified MSP or GME-EDC-modified MSP. (A) The results of a typical 24 kDa binding experiment. The Western blot is shown for both the unmodified and modified MSPs, with the 24 kDa protein being detected immunologically. The amount of added 24 kDa protein is shown above (mole/mole PS II). The amount of 24 kDa protein which was bound was determined by digitization and quantification of these Western blots and is plotted in panel B. (B) Plot of the normalized relative density of the immunologically detected 24 kDa protein vs 24 kDa protein added (mole/mole PS II). Filled squares, 24 kDa protein binding to unmodified MSP-reconstituted PS II membranes; filled circles, 24 kDa protein binding to modified MSP-reconstituted PS II membranes. Error bars are $\pm$ 1.0 standard error, $n = 5$. Please note that some error bars are smaller than the data point symbols.

Table 1: Assignments for Peptides Produced from Either Trypsin or *Staphylococcus* V8 Protease Digestion of the MSP Which Was Modified with GME-EDC on NaCl-Washed PS II Membrane

observed mass[a]	predicted mass[b]	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.	^{152}G−^{159}K
1254.10	1253.39	0.71	ca.	^{5}R−^{14}K
1389.63	1388.71	0.92	ca.	^{50}F−^{60}K + 1 VP[d]
1626.02	1624.78	1.24	ca.	^{1}E−^{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[g]	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] Data were originally published in ref *26* and are being reevaluated here with respect to 24 kDa protein binding. [b] Mass predicted from analysis of the *psbO* gene (*37*). 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. [g] Monoisotopic mass; all others are average masses.

MSP or saturating amounts of the modified MSP. The 24 kDa protein reconstitutes fully to PS II membranes which contained control MSP, exhibiting specific binding which saturates at 1−2 mol of the 24 kDa protein per mole PS II. Additionally, only a small amount of nonspecific binding is also observed. These results are very similar to those which have been obtained previously by other investigators (*34*−*36*). A significantly different binding profile for the 24 kDa protein is observed if the PS II membranes contained the modified MSP. These membranes exhibited a very low capacity for 24 kDa protein binding. It must be emphasized here that both the control MSP-containing membranes and the modified MSP-containing membranes were fully saturated with their respective MSPs. Since the GME-EDC modification(s) present on the modified MSP had essentially no effect on its binding to the photosystem (*26*), the defective binding observed for the 24 kDa onto membranes containing the modified MSP must be a result of the modification of carboxylates on the MSP which do not interfere with MSP binding to the photosystem.

In an effort to identify which labeled domains on the MSP are responsible for the observed effects on 24 kDa protein binding, we have utilized protease digestion and MALDI mass spectrometry to identify the regions of the MSP which are labeled with GME-EDC. Table 1 lists the peptides obtained by digestion of the MSP with trypsin and V8 protease and their assignments. 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 different matrixes, which differentially mobilize the proteolytic peptides into the vapor phase, greatly increases the number of peptides which can be analyzed in these experiments. Increasing the number of peptides which 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 the modified MSP 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. 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 (*37*). It should be noted that native MSP does not appear to contain any mass-altering posttranslational modifications. MALDI mass spectrometry indicates a mass of 26 523.4 Da (*38*), which has been verified by other workers (*39*, *40*). This is virtually identical (within the error of the mass spectrometer) to the predicted mass of 26 531.6 Da predicted from its derived amino acid sequence (*37*). The program GPMAW ver. 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 while associated with NaCl-washed PS II membranes. This modification resulted in the addition of 72.09 Da for each modified carboxylate group. Second, prior to proteolytic 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 (*41*), and added 105.14 Da per modified cysteine.

The GME-EDC-modified MSP contained eight tryptic peptides and three *Staphylococcus* V8 protease peptides which contained the modification. Since each MSP which had been modified on the membrane contained one to two

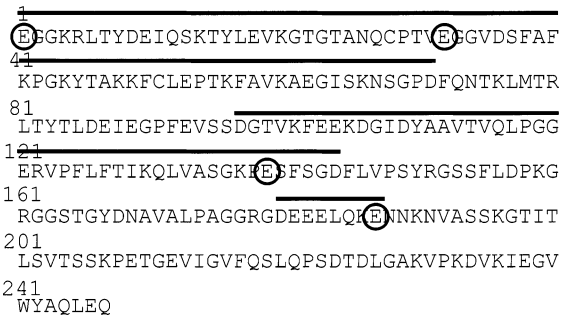

FIGURE 3: The sequence of spinach MSP. Superior lines indicate domains modified in the GME-EDC reaction when the MSP was associated with NaCl-washed PS II membranes. Carboxylates modified in these domains are responsible for the loss of efficient 24 kDa protein binding. The circled amino acids are at positions where acidic residues are absolutely conserved in all higher plant MSPs but which are poorly conserved in the cyanobacterial MSPs. We hypothesize that these residues may be the modification sites responsible for loss of 24 kDa protein binding.

modified carboxylates (*26*), 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 (*31*), N-succinimidyl propionate, and 2,4,6-trinitrobenzene sulfonic acid (*27*).

Our results indicate that the domains ^{1}E−^{71}D, ^{97}D−^{144}D, and ^{180}D−^{187}E can be labeled when the MSP is associated with NaCl-washed PS II membranes. The results of these experiments are summarized in Figure 3. A word of caution is necessary in the interpretation of these results. It is possible that the removal of the 24 and 16 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.

It is clear that modification of carboxylates in the domains ^{1}E−^{71}D, ^{97}D−^{144}D, and ^{180}D−^{187}E of the MSP has a profound effect on the ability of PS II to bind the 24 kDa protein. A number of hypotheses 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 24 kDa protein. While charge-pair interactions have never been observed between the MSP and the 24 kDa protein using EDC cross-linking (a zero-length cross-linker), it should be noted that if the charge-pair is buried at the face of the interaction it would not be identified by this technique. A second possibility is that the conjugation of glycine methyl ester to the MSP carboxylates sterically interferes with the association of the 24 kDa protein to PS II. The presence of the methyl glycine moiety may prevent the formation of other protein−protein interactions (either between MSP and the 24 kDa protein or between the 24 kDa protein and other PS II components) which are required for effective binding of the 24 kDa to PS II. Finally, it is possible that binding of the MSP elicits conformational changes in other PS II components which are required for efficient 24 kDa protein binding. GME-EDC modification of the MSP may, either directly or indirectly, prevent these putative conformational changes from occurring. It is impossible at this point in time to differentiate between these (and possibly other) mechanisms.

The MSP is an acidic protein with a pI of about 5.2 and contains 35 carboxylates. The domains which we have demonstrated to be labeled by GME-EDC (^{1}E−^{71}D, ^{97}D−^{144}D, and ^{180}D−^{187}E) contain 22 acidic residues. Which of these residues are required for efficient 24 kDa protein binding? While, at this time, we cannot address this question directly (experiments are currently underway), an analysis of the sequences of the MSPs from eleven higher plants and eight cyanobacteria (*42*) allows us to hypothesize which residues may be involved. Since higher plant MSP can functionally rebind to cyanobacterial PS II and cyanobacterial MSP can functionally rebind to higher plant PS II, it appears that the majority of the binding determinants for MSP to PS II have been conserved evolutionarily (*21*, *43*). Additionally, all higher plants possess the 24 kDa protein while this component appears to be replaced by cytochrome c_{550} in the cyanobacteria. We would argue, therefore, that residues which were highly conserved in the higher plant MSPs but which were poorly conserved in cyanobacterial MSPs would be candidates for residues whose modification could interfere with the interaction of MSP with the 24 kDa protein. Only four such acidic residues are found upon examination of the sequence information: ^{1}E, ^{32}E, ^{139}E, and ^{187}E. ^{1}E is in a domain of the MSP which has been implicated in its association with the intrinsic proteins of the photosystem (*11*−*14*, *44*). Our results indicate that it may also be involved in the interaction of the 24 kDa protein with the photosystem (either directly or indirectly, see above). More interestingly, ^{32}E, ^{139}E, and ^{187}E are residues which had been previously predicted to be exposed to the bulk solvent on NaCl-washed PS II membranes (*6*). This prediction was based on a global analysis of the secondary structure of the MSP, site-directed mutagenesis experiments performed in both the cyanobacterial and higher plant systems, and protein chemical modification and cleavage studies performed in higher plants. We believe that these are possible candidates for sites of GME-EDC modification which disrupts 24 kDa protein binding. Studies are ongoing to determine which of these residues (or others) are responsible for the profound effects on 24 kDa protein binding which we have reported in this communication.

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