Binding of Manganese Stabilizing Protein to Photosystem II: Identification of Essential N-Terminal Threonine Residues and Domains that Prevent Nonspecific Binding †

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Binding of Manganese Stabilizing Protein to Photosystem II: Identification of Essential N-Terminal Threonine Residues and Domains that Prevent Nonspecific Binding[†]

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ABSTRACT: The N-terminus of spinach photosystem II manganese stabilizing protein (MSP) contains two amino acid sequences, [4]KRLTYD[10]E and [15]TYL[18]E, that are necessary for binding of two copies of this subunit to the enzyme [Popelkova et al. (2002) *Biochemistry 41*, 10038−10045]. To better understand the basis of MSP−photosystem II interactions, the role of threonine residues in the highly conserved motifs T(Y/F)DE and TY has been characterized. Deletion mutants lacking the first 5, 6, 7, and 15 amino acid residues at the N-terminus of the protein were examined for their ability to reconstitute activity in MSP-depleted photosystem II. The results reported here show that truncations of five and six amino acid residues (mutants ΔR5M and ΔL6M mutants) have no negative effect on recovery of oxygen evolution activity or on binding of MSP to photosystem II. Deletion of seven residues (mutant ΔT7M) decreases reconstitution activity to 40% of the control value and reduces functional binding of the mutant protein to photosystem II from two to one copy. Deletion of 15 amino acid residues (mutant ΔT15M) severely impairs functional binding of MSP, and lowers O_2 evolution activity to less than 20% of the control. ΔT7M is the only mutant that exhibited neither nonspecific binding to photosystem II nor changes in tertiary structure. These, and previous results, show that the highly conserved Thr7 and Thr15 residues of MSP are required for functional binding of two copies of the eukaryotic protein to photosystem II. Although the N-terminal domains, [1]EGGKR[6]L, [8]YDEIQS[14]K, and [16]YL[18]E of spinach MSP are unnecessary for specific, functional binding interactions, these sequences are necessary to prevent nonspecific binding of the protein to photosystem II.

Chlorophyll-containing organisms produce molecular oxygen as a byproduct of water oxidation, which takes place in the oxygen evolving complex (OEC)[1] of photosystem II (PSII). In eukaryotes, three extrinsic proteins (17, 23, and 33 kDa) provide a structural framework for retention and stabilization of the essential inorganic cofactors (Ca^+, Cl^-, and a cluster of four Mn atoms (*1*)) that form the active site of the OEC. The 33-kDa protein, also known as manganese stabilizing protein (MSP), functions to stabilize binding of the tetranuclear manganese cluster to the OEC, and to accelerate the rate of O_2 evolution activity (*2−6*). It has also been suggested that MSP regulates the Mn−Cl^- interaction by facilitating Cl^- binding to the OEC (*7*), and the presence of MSP is required for binding of the 23-kDa protein to PSII (*8*). A direct role for MSP in Mn redox reactions (deprotonation of glutamate and/or aspartate residues during water oxidation) has been proposed as well (*9*).

The 17, 23, and 33 kDa proteins can be selectively extracted from intact PSII membranes (BBY). Washing intact PSII preparations in 2 M NaCl releases the 17- and 23-kDa proteins along with Ca^{2+} and Cl^-; addition of these cofactors restores substantial amounts of activity (*10*). A subsequent exposure of the salt-washed preparation to 2−3 M urea specifically extracts MSP, which lowers O_2 evolution activity and destabilizes binding of the Mn cluster to PSII (*3, 11*). When the *psbO* gene encoding MSP is deleted in the cyanobacterium *Synechocystis sp.* PCC6803, oxygen evolution activity is also lowered (*12, 13*). In the green alga *Chlamydomonas*, however, deletion of the *psbO* gene produces cells that are unable to grow photoautotrophically and to accumulate other PSII subunits (*14*).

Interactions of MSP with the E loop of the CP47 subunit of PSII have been documented (*15−19*). Although results of three-dimensional cryoelectron microscopy of spinach PSII

[1] Abbreviations: CD, circular dichroism; Chl, chlorophyll; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IPTG, isopropyl-*β*-D-thiogalactopyranoside; MSP, manganese stabilizing protein; OEC, O_2 evolving complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TOPO, plasmid for gene expression with T7 RNA polymerase; PS, photosystem; *psbO*, gene encoding precursor MSP; SDS, sodium dodecyl sulfate; sw-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic proteins; TMACl, tetramethylamonium chloride; usw-PSII, urea salt-washed photosystem II membranes depleted of 33, 23, and 17 kDa extrinsic proteins; UV, ultraviolet; Δ, represents missing amino acid residues; 2°, secondary structure of protein; 3°, tertiary structure of protein.

supercomplexes are interpreted as resolving a single copy of MSP per PSII (*20*, *21*), transmission electron microscopy of these PSII preparations detects one or two MSP subunits within a globular space on the surface of PSII (*22*). Other studies indicate that two copies of MSP are bound per PSII reaction center. This stoichiometry is based on a number of observations (*23*−*30*): quantification of PSII-bound MSP (*23*, *24*), analyses of binding of MSP to PSII (*25*−*29*), and the detection of two distinct peptide fragments as a result of the trypsin cleavage of intact PSII membranes. The smaller fragment is recognized by antibodies directed against both the MSP N-terminus and the entire MSP sequence (anti-EMSP), while the larger fragment is recognized only by anti-EMSP, indicating a cleavage of two separate MSP molecules (*30*).

Eaton-Rye and Murata employed proteolytic digestion to show that 16 or 18 N-terminal amino acid residues of MSP are important for its functional binding to PSII (*31*). This proposal was substantiated and extended by Odom and Bricker, who used cross-linking by EDC to show that the [1]E-[76]K domain of MSP interacts with the [364]E-[440]D domain of the large extrinsic E loop of CP47 (*17*). When an N-terminal mutation (D9K) was introduced in *Synechocystis* PCC6803, only slightly altered O_2 evolution activity relative to wild type was observed (*32*). Site-directed mutants D9K and D9N, replacing the highly conserved aspartate residue at position 9, in spinach MSP produced the same oxygen evolution activity as was observed with the wild-type protein (*33*). Recent studies using deletion mutations at the N-terminus of spinach MSP have shown that there are two N-terminal domains, [4]KRLT(Y/F)D[10]E and [15]TY(L/M)[18]E, that are required for binding of two copies of MSP to PSII. Alignment of all available MSP N-terminal sequences from various organisms has revealed that these domains contain the highly conserved amino acid motifs T(Y/F)DE and TY in eukaryotes, but only one highly conserved motif, TY, is found in prokaryotes (*34*, *35*).

Several studies have demonstrated that negatively charged carboxyl groups of aspartyl (D) and glutamyl (E) residues and/or positively charged amino groups of lysine (K) and arginine (R) residues are involved in electrostatic interactions between MSP and PSII (*27*, *36*−*38*). However, the highly conserved TYDE and TY motifs in the binding-related domains of eukaryotic MSP start with uncharged threonine residues. To determine whether these threonines play a role in MSP binding, new MSP mutants were prepared by deletion of 5, 6, 7, and 15 amino acid residues in the spinach MSP sequence using site-directed mutagenesis. Truncation by five and six amino acids at the MSP N-terminus had no effect on O_2 evolution activity compared to wild type. Deletion of seven and 15 N-terminal residues, however, reduced activity reconstitution to 40 and <20% of the control values, respectively. Plotting of activity restoration as a function of bound MSP showed that the ΔR5M and ΔL6M mutants exhibited wild-type levels of specific binding (2 mol of MSP/mol PSII). For ΔT7M and ΔT15M, the same plotting methods showed that 1 mol of ΔT7M per mol of PSII and only small fraction of ΔT15M were specifically bound to PSII. All mutants except ΔT7M also bound nonspecifically at the higher protein concentrations used to generate the binding curves for quantitative analyses of MSP reconstitution of PSII; these mutants also exhibited modifications of

their tertiary structure. Combining the results presented here with those from refs *34* and *35* shows that Thr7 and Thr15 are critical amino acid residues which are required for functional binding of two copies of eukaryotic MSP to PSII. In addition, the N-terminal domains [1]EGGKR[6]L, [8]YDEIQS[14]K, and [16]YL[18]E are important components of the tertiary structure of wild-type MSP, which function to optimize functional binding of MSP, and at the same time prevent nonspecific binding of MSP to PSII.

MATERIALS AND METHODS

Mutations of psbO cDNA and Transformations of Escherichia coli Cells. For construction of cDNA encoding the recombinant deletion mutants ΔR5M, ΔL6M, ΔT7M, and ΔT15M, the oligonucletides 5′-TCATATGTTGACCTAC-GACGAGATC-3′, 5′-TCATATGACCTACGACGAGATC-CAG-3′, 5′-TCATATGTACGACGAGATCCAGAGC-3′, and 5′-TCATATGTACCTCGAAGTCAAAGGAACT-3′, respectively, were synthesized (Gibco BRL or Invitrogen custom primers) on the basis of the sense strand of the MSP N-terminal coding region. The oligonucleotide for an anti-sense strand of the C-terminal coding region was the same as that described in ref *35*. After amplification of the coding region by PCR using these oligonucleotides, PCR products were subcloned into the pCR T7/CT TOPO vector (Invitrogen) and the product of the cloning reaction was transformed into TOP 10 F′ cells (Invitrogen). The correct orientation of the PCR fragment in the TOPO vector was confirmed and the DNA sequence was verified. The TOPO *psbO* construct was then transformed into BL21(DE3)pLysS *E. coli* cells. N-terminal protein sequencing of the first five amino acids by Edman degradation was used to confirm use of the correct reading frame during protein translation. The expected sequences MLTYD (for ΔR5M), MTYDE (for ΔL6M), MYDEI (for ΔT7M), and MYLEV (for ΔT15M) were obtained.

Overexpression and Purification of Recombinant MSPs. Procedures for overexpression and purification of recombinant MSPs are described in ref *35*, except that two different concentrations of IPTG were used for overexpression of recombinant mutant proteins in *E. coli*: 0.5 mM for ΔR5M and ΔT7M and 1 mM for ΔL6M and ΔT15M. After extraction of recombinant proteins from inclusion bodies (*34*) and overnight incubation in 3 M urea, 5% betaine (w/v), 20 mM Tris (pH 8), 5 mM NaCl at 4 °C, proteins were purified using a Pharmacia Resource Q column equilibrated with the same buffer and a linear gradient (5−250 mM NaCl). Recombinant MSP eluted between 120 and 150 mM NaCl. After purification, recombinant mutants were dialyzed in Tris buffer (100 mM Tris, 10 mM NaCl, pH 8) and then MES buffer (50 mM MES, 10 mM NaCl, pH 6). 0.4 mM sucrose was added to each protein before storage at −70 °C. The concentration of MSP was determined spectrophotometrically using the extinction coeficient of 16 mM^{-1} at a wavelength of 276 nm.

Reconstitution of PSII with MSP, Functional Analysis. Photosystem II membranes were isolated and extrinsic proteins were extracted as described in ref *34*. Polypeptide depleted PSII membranes were reconstituted with recombinant MSP in a solution containing 37 mM MES (pH 6), 10 mM $CaCl_2$, 80 mM NaCl, 100 μg BSA/mL, 0.3 M sucrose,
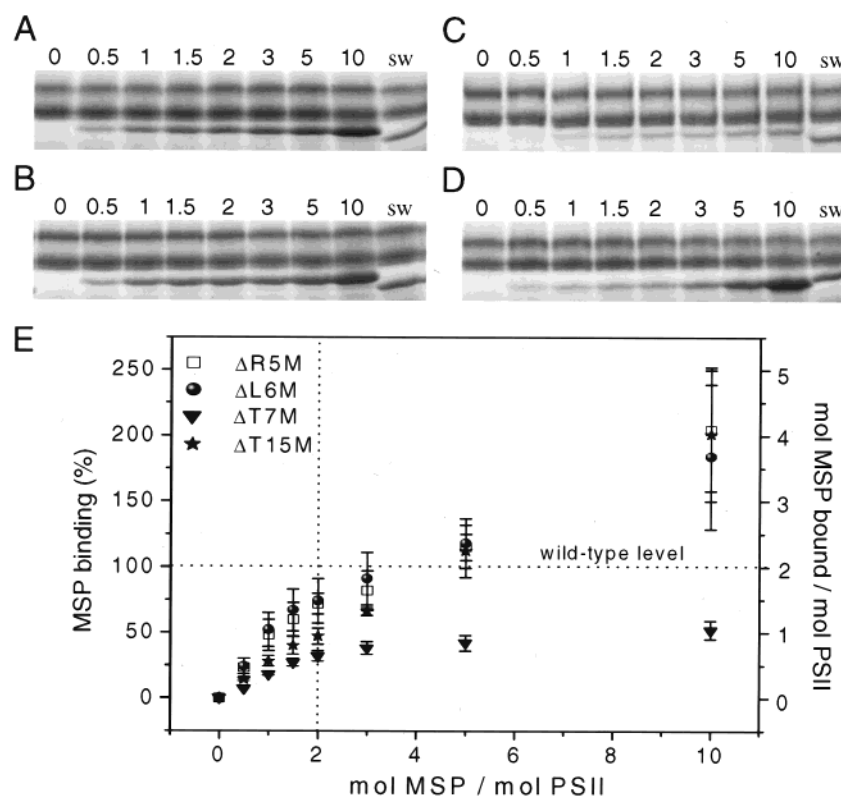
FIGURE 1: Binding of ΔR5M, ΔL6M, ΔT7M, and ΔT15M to PSII. The Coomassie blue stained SDS−PAGE gel shows usw-PSII samples reconstituted with ΔR5M (A), ΔL6M (B), ΔT7M (C), and ΔT15M (D), respectively, for 1 h at room temperature. The protein bands shown on the gel, from top to bottom, are CP47, CP43, and MSP. The mol MSP/mol PSII used for reconstitution are indicated at the top of the lanes; sw, control sw-PSII. (E) Binding curves for usw-PSII reconstituted with ΔR5M, ΔL6M, ΔT7M, and ΔT15M, respectively. SDS−PAGE gels of reconstituted samples were analyzed by densitometry. Binding efficiency of ΔR5M, ΔL6M, ΔT7M, and ΔT15M was expressed as a function of added MSP (mol/mol of PSII; *x* axis), both as a fraction of MSP bound to the sw-PSII control (100% left *y* axis) and as the amount of bound mol of MSP/mol of PSII (right *y* axis). Each point is the average of three separate experiments and vertical bars represent the standard deviation. Binding data for WT MSP are omitted for purposes of clarity in this and the following figures, and replaced by the dotted lines, which define the end points of activity reconstitution and binding stoichiometry.

and 2% betaine (w/v). The artificial acceptor DCBQ (600 μM) was added to the buffer during O_2 evolution assays. To determine the extent of rebinding of MSP to PSII, unbound MSP was removed by centrifugation at 12000*g* at 4 °C for 10 min. The pellet was washed in 800 μL of SMTC buffer (50 mM MES (pH 6), 20 mM $CaCl_2$, 60 mM TMACl, and 0.4 M sucrose), recentrifuged, and then resuspended in 60 μL of SMTC to obtain a final concentration of 1 mg of Chl/mL. Samples were analyzed by SDS−polyacrylamide gel electrophoresis. The amount of MSP bound to PSII was estimated by integration of the Coomassie-stained MSP bands using Sigmagel software (Jandel Scientific), based on the linear relationship between Coomassie staining intensity and MSP concentrations used in the experiments reported here. Other experimental details are reported in refs *34* and *35*.

Structural Properties of MSP Mutants. Recombinant mutant proteins were dialyzed in 10 mM KH_2PO_4 (pH 6) and diluted to a concentration of 10 μM. The CD spectra were measured using an AVIV 62 DS CD spectrometer calibrated with (+)10-camphorsulfonic acid disolved in H_2O (1 mg/mL). Experimental conditions are shown in the legend of Figure 4. For far-UV CD spectra, a 250−178 nm scan width and a 300 μL sample volume were used. The CONTINLL and CDSSTR methods from the CDPro package (http://lamar.colostate.edu/~sreeram/CDPro) were used for analysis of far-UV CD spectra. Size-exclusion chromatography was carried out using Superose-12 column on a

Pharmacia FPLC system calibrated with 20 mM bis-Tris (pH 6.4) and 150 mM NaCl as described in ref *34*.

RESULTS

To better characterize the interactions that promote binding and assembly of MSP into the OEC, new site-directed mutants (ΔR5M, ΔL6M, ΔT7M, and ΔT15M) were prepared by placing Met residues (using the translation initiation codon) at positions 5, 6, 7, and 15 in the N-terminal sequence of mature MSP. These mutants were characterized with respect to their ability to restore O_2 evolution activity, and were analyzed for changes in secondary and tertiary structure in solution. Last, results of these experiments were combined with earlier data to identify amino acid residues that are essential for specific binding interactions between MSP and PSII.

Functional Characterization of ΔR5M, ΔL6M, ΔT7M, and ΔT15M Mutants. Figure 1 presents data on rebinding of the mutant proteins to usw-PSII. As can be seen from panels A, B, C, and D, the ΔR5M, ΔL6M, and ΔT7M mutants migrated more slowly than wild type on SDS−PAGE, while ΔT15M migrated faster under the same electrophoresis conditions. These differences in electrophoretic mobility have been observed with other MSP mutants (*34, 35*). There is no simple explanation for this phenomenon, because the shifts in mobility do not correlate with reductions in the molecular mass of MSP (see below) as a result of elimination
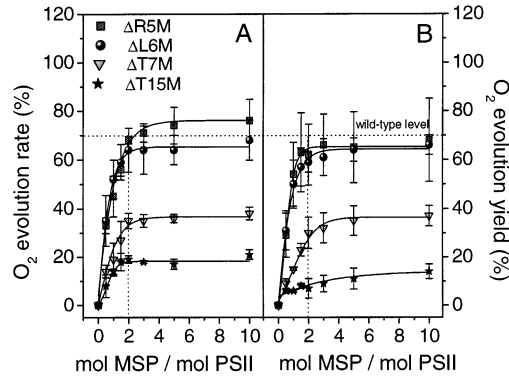
FIGURE 2: Recovery of O_2 evolution activity by usw-PSII membranes reconstituted with $\Delta R5M$, $\Delta L6M$, $\Delta T7M$, and $\Delta T15M$, respectively. (A) O_2 rate and (B) O_2 yield assayed during 1 and 4 min of continuous illumination, respectively, plotted as a function of mol of MSP added to reconstitution mixtures. Samples were assayed after incubation of reconstitution mixtures for 1 h at room temperature. The residual activity of usw-PSII was subtracted from the activities of reconstituted samples. 100% corresponds to the activity of control sw-PSII (350 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}). Points are averages of three experiments and the vertical bars at each point give the standard deviation. For wild type (dotted lines), see refs *26* or *34*.

of amino acids. For example, the truncation mutants $\Delta R5M$, $\Delta L6M$, and $\Delta T7M$ exhibited decreased, rather than increased, mobility on SDS−PAGE. This may be a consequence of the natively unfolded behavior of the protein (*39*, *40*). Figure 1E shows the results of experiments using gel scanning to produce a quantitative evaluation of protein binding to PSII. Dotted lines are included to depict the binding behavior of wild-type MSP, which saturates at 2 mol of MSP/mol of PSII, equivalent to binding of 2 mol of native MSP in sw-PSII membranes (100%). Binding of $\Delta R5M$, $\Delta L6M$, and $\Delta T15M$ increased gradually and failed to saturate, even at the higher protein concentrations present in the reconstitution mixtures. About 1.5 mol of $\Delta R5M$ or $\Delta L6M$/mol of PSII and 0.9 mol of $\Delta T15M$/mol of PSII were bound when 2 mol of mutant MSP/mol of PSII was present in the incubation mixtures used for reconstitution. For $\Delta R5M$, $\Delta L6M$, or T15M, about 4 mol of mutant MSP/mol of PSII was bound when 10 mol of each mutant/mol of PSII was used for the reconstitution incubation step. A similar type of binding behavior was previously detected for the deletion mutants $\Delta G3M$, $\Delta S13M$, and $\Delta K14M$ (see refs *34* and *35*). Binding of the $\Delta T7M$ mutant, on the other hand, exhibited saturation behavior. About 0.6 mol of $\Delta T7M$/mol of PSII was bound at 2 mol of $\Delta T7M$/mol of PSII added to the reconstitution mixture and only 1 mol of $\Delta T7M$/mol of PSII was bound if 10 mol of $\Delta T7M$/mol of PSII was included in the incubation mixtures used for reconstitution.

Figure 2 presents data on the reconstitution of oxygen evolution activity, using rate and yield assays, after incubation of usw-PSII with different concentrations of the various deletion mutants. The effect of rebinding of the mutant proteins on activities, plotted as a function of the amount of MSP actually bound to PSII, is depicted in Figure 3. As can be seen, the $\Delta R5M$ and $\Delta L6M$ mutants produced reconstitutions of activity similar to that observed for wild-type MSP (saturation at 70% of the control value upon binding of 2 mol of MSP/mol of PSII; see dotted lines in Figures 2 and 3). Binding of 2 mol of $\Delta R5M$ or $\Delta L6M$/mol of PSII produced maximal recovery of activity (around 70% of the
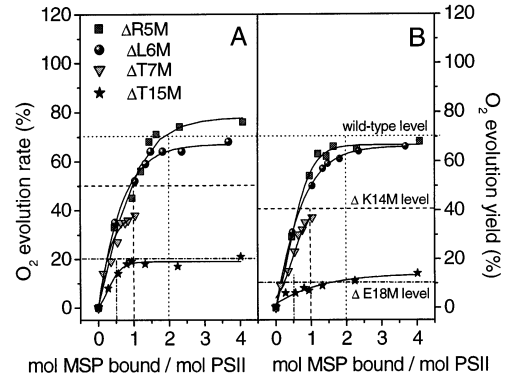

FIGURE 3: O_2 evolution rates (A) and yields (B) restored by reconstitution of usw-PSII membranes with mutants $\Delta R5M$, $\Delta L6M$, $\Delta T7M$, and $\Delta T15M$, respectively, and plotted as a function of the amount of MSP that is actually bound to usw-PSII. Dotted, dashed, and dash−dotted lines indicate reconstitution activity of wild type, $\Delta K14M$, and $\Delta E18M$, respectively (data from refs *34* and *35*). For reconstitution conditions and calculation of 0 and 100% activity, see the legend of Figure 2.

Table 1: Additional Binding of MSP Deletion Mutants to Intact PSII Membranes[a]

addition to reconstitution mixtures (mol MSP/mol intact PSII)	additional binding to intact PSII (%)				
	$\Delta R5M$	$\Delta L6M$	$\Delta T7M$	$\Delta E10M$	$\Delta T15M$
2	16	19	0	4	3
5	30	24	0	24	19
10	68	23	4	41	51

[a] The native MSP content of intact PSII membranes was used as the control (100%) protein level. The data are presented as a percentage of the control; staining intensity of CP47 was used as an internal standard for normalization of the MSP band.

control), which indicates that additional binding (up to 2 mol of $\Delta R5M$ or $\Delta L6M$/mol of PSII) was nonspecific. Recovery of O_2 evolution activity by reconstitution with $\Delta T7M$ attained about 40% of the control value when 1 mol of $\Delta T7M$/mol of PSII was bound. Finally, binding of about 0.5 mol of $\Delta T15M$/mol of PSII reconstituted about 20% of the control O_2 evolution rate and 10% of the control O_2 evolution yield. As the data of Figures 2 and 3 show, additional binding of $\Delta T15M$ (up to 4 mol of $\Delta T15M$/mol of PSII) failed to contribute to any further increase in activity. These data show that only a small fraction of $\Delta T15M$ can bind functionally, and that most of the protein is probably associated with PSII as a result of the nonspecific binding shown in Figure 1E.

It has been previously shown (*34*, *35*) that certain deletion mutants of MSP are capable of nonspecific binding to PSII, and this was verified for the present group of mutants by conducting binding experiments with intact PSII membranes, which possess native MSP bound to specific sites with high affinity. In this case, only nonspecific binding sites are available. As the data presented in Table 1 show, the $\Delta R5M$, $\Delta L6M$, and $\Delta T15M$ mutants are able to bind nonspecifically to PSII. About 1 mol of $\Delta R5M$ or $\Delta T15M$/mol of intact PSII, and about 0.5 mol of $\Delta L6M$/mol of intact PSII was bound upon incubation of native PSII preparations with 10 mol of mutant MSP/mol of intact PSII, followed by washing to remove any weakly bound MSP. The $\Delta T7M$ mutant did not exhibit nonspecific binding under similar conditions.
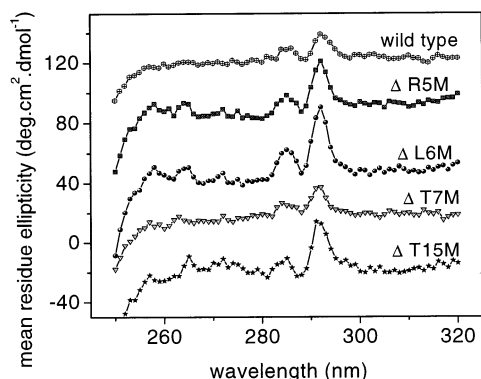
FIGURE 4: Near-UV CD spectra (protein solution minus buffer) of wild type, ΔR5M, ΔL6M, ΔT7M, and ΔT15M. Each spectrum is the average of 20 scans. Experimental conditions were as follows: time constant, 1 s; bandwidth, 1 nm; scan step, 1 nm; room temperature; scan width, 320−250 nm; path length, 1 cm; sample volume, 1 mL; protein concentration, 10 μM in 10 mM potassium phosphate buffer, pH 6.

Solution Structure Properties of ΔR5M, ΔL6M, ΔT7M, and ΔT15M. All mutant proteins characterized in this study (as well as those presented in refs *34* and *35*) were in monomeric form, since single UV absorption peaks were recorded during elution of the proteins on an FPLC gel filtration column. The apparent molecular masses of truncation mutants exhibited small decreases in mass relative to wild type (37 kDa), consistent with increasing extents of N-terminal deletion of the MSP amino acid sequence; molecular masses of 34.4, 34.5, 34.3, and 33.2 kDa were observed for the ΔR5M, ΔL6M, ΔT7M, and ΔT15M mutants, respectively, using a calibrated Superose 12 FPLC column.

Partial information about the tertiary structure of MSP can be obtained from near-UV CD spectra. The amplitudes of characteristic peaks of near-UV CD spectra due to aromatic amino acid residues are indicators of the hydrophobicity of the environment surrounding these species (*34*, *35*). Figure 4 shows that the peaks at 285 nm (Tyr) and 292 nm (Trp) in the near-UV CD spectra of ΔR5M, ΔL6M, and ΔT15M are more prominent than the corresponding peaks observed in near-UV CD spectra of wild-type MSP. The near-UV CD spectrum of the ΔT7M mutant was very similar to that of wild type (Figure 4). Changes in the characteristic peaks of near-UV CD spectra were in agreement with the appearance of a distinct 293 nm shoulder from Trp in UV absorption spectra, which also monitors solution tertiary structure of the protein (data not shown). Comparisons of near-UV CD spectra (Figure 4), binding curves (Figure 1), and data from binding experiments with intact PSII (Table 1) indicate that a modification of the tertiary structure of the mutants shown here, which increases the hydrophobicity of the domains around Tyr and Trp, also enhances the ability of these proteins to occupy nonspecific binding sites on PSII.

Total content of α-helices, β-sheets, and random coils in the secondary structure of deletion mutants was also analyzed using the far-UV CD spectra of ΔR5M, ΔL6M, ΔT7M, and ΔT15M (data not shown); the same programs and basis sets as described in refs *34* and *35* were used. No significant changes in the estimated secondary structure contents of these mutants were observed relative to wild type (data not shown). This result is very similar to that obtained for other truncation

mutants, presented in refs *34* and *35*, with the exception of the ΔE18M mutant.

DISCUSSION

It was recently shown that two N-terminal sequences, [4]-KRLT(Y/F)D[10]E and [15]TY(L/M)[18]E, of MSP are required for binding of two copies of the eukaryotic protein to PSII (*34*, *35*). Alignment of all available N-terminal sequences of eukaryotic MSPs shows that the first and second sequences involved in binding of the protein to PSII contain the highly conserved motifs T(Y/F)DE and TY, respectively. To probe these sequences in more detail and to attempt to identify essential amino acids that participate in the MSP−PSII interaction, we have focused on the uncharged Threonine residues. The approach, applied successfully in other studies on the N-terminus of MSP, was to employ site-directed mutagenesis to produce new MSP mutants, ΔR5M, ΔL6M, ΔT7M, and ΔT15M.

Deleting five or six amino acid residues from the MSP N-terminus (ΔR5M and ΔL6M mutants) produced an effect that is essentially similar to what has been observed after deleting three residues (mutant ΔG3M (*34*)). Both mutants produced reconstitutions of activity that were very similar to those obtained with wild-type MSP, namely, an activity recovery of about 70% of the control value upon rebinding of 2 mol of MSP/mol of PSII. However, as is shown in Figure 1, these mutants were also capable of additional, nonspecific binding of up to 2 mol of the protein to PSII at the highest protein concentrations used for reconstitution with MSP depleted PSII (Figures 1−3, and Table 1). All protein solutions used in these experiments were centrifuged before use in reconstitution experiments to eliminate any artifacts that might be caused by protein aggregation. The results obtained with the ΔR5M and ΔL6M mutants establish that the sequence [7]TYD[10]E, from the previously identified sequence [4]KRLTYD[10]E, is required for MSP binding.

A substantial change in MSP binding and activity reconstitution was induced by truncation of seven N-terminal amino acids (mutant ΔT7M). Binding of this mutant to a level of 1 mol of ΔT7M/mol of PSII restored activity to a level of 40% of the control (salt-washed PSII) value (Figures 1−3). This result is very similar to what was observed with mutants ΔE10M, ΔS13M, and ΔK14M reported in refs *34* and *35* (see dashed lines in Figure 3); all three of these mutants were capable of functional binding of 1 mol of MSP/mol of PSII, in each case with an accompanying restoration of O_2 evolution activity of between 40 and 50% of the sw-PSII control. The ΔT7M mutant was unable to bind nonspecifically to PSII, as is indicated by the shape of binding curve, which shows saturation behavior (Figure 1), and by its inability to bind to intact PSII, where specific binding sites are occupied by native MSP (Table 1).

Deletion of the first 15 amino acid residues from the MSP N-terminus, which produced the ΔT15M mutant, severely impaired the protein's function. Although binding of ΔT15M to usw-PSII could be detected at high levels (up to 4 mol of ΔT15M/mol of PSII when 10 mol of ΔT15M/mol of PSII was added to the reconstitution mixture), binding failed to saturate, and this mutant was incapable of producing reconstitutions of activity in excess of about 10−20% of the control (compare Figures 1−3). Experiments with intact PSII

Table 2: Binding Affinities and Protein Solution Structure Properties of Spinach Wild Type, Precursor, and N-Terminal Deletion Mutants of MSP

protein	binding (mol MSP/mol PSII)		reconstitution of activity (% of wt values)	structural changes		ref
	specific	nonspecific[a]		3° structure[b]	2° structure	
wild type	2	0	100			(26, 34)
preMSP	2	1	100	↓	none	(34)
ΔG3M	2	4	130	↑	none	(34)
ΔR5M	2	2	100	↑	none	this study
ΔL6M	2	2	95	↑	none	this study
ΔT7M	1	0	60	none	none	this study
ΔE10M	1	3	70	↑	none	(34), this study
ΔS13M	1	3	70	↑	none	(35)
ΔK14M	1	3	70	↑	none	(35)
ΔT15M	>0	<4	20	↑	none	this study
ΔE18M	>0	<1	20	↓	yes	(35)

[a] Maximal nonspecific binding with 10 mol of mutant MSP/mol of PSII used for reconstitution of usw-PSII with mutant MSP; [b] Arrows indicate an increase or decrease in the hydrophobic environment around aromatic amino acid residues.

revealed that this mutant also possesses a high capacity for nonspecific binding (Table 1). Thus, only a minor fraction of ΔT15M seems to bind specifically to usw-PSII, a functional behavior that is very similar to what was reported for mutant ΔE18M in ref *35* (see dash−dotted lines in Figure 3).

The functional and structural attributes of all N-terminal alterations of wild-type MSP reported here and in refs *34* and *35* are summarized in Table 2. Specific binding of these proteins to PSII and their ability to reconstitute activity (see second and fourth column in Table 2) indicate that either addition of the transit peptide to the N-terminus of MSP, or elimination of the ^{1}EGGKR^{6}L sequence from the N-terminus of the mature spinach protein have little, if any, negative effect on MSP rebinding and function. Removal of the ^{7}T amino acid residue by deletion of the ^{1}EGGKRL^{7}T sequence from the MSP N-terminus, however, reduces binding from 2 to 1 copy of MSP per PSII reaction center, and decreases activity to about 60% of that of the wild-type protein. This same defect can be obtained for any truncation from ^{7}T up to amino acid residue 14 (Table 2, refs *34* and *35*). A further deletion of 15 or 18 amino acid residues dramatically impairs functional binding of the second MSP copy to PSII, and reduces the ability of these mutant proteins, when they do rebind, to reconstitute activity maximally to about 20% of wild type levels. These data show that threonines at positions 7 and 15 are critical amino acid residues that are essential for the functional binding of two eukaryotic MSP molecules to PSII. Although positively and/or negatively charged residues have been proposed to be important for the MSP−PSII interaction (*27, 36*), the discovery that uncharged Thr residues play a role in PSII binding of MSP is consistent with findings of Burnap et al. (*32*) and Seidler et al. (*33*), who showed that a highly conserved amino acid residue, ^{9}D, at the MSP N-terminus is not required for high rates of O_2 evolution activity, nor for MSP binding to PSII. Involvement of threonines in protein function and protein−protein interactions is not unique to MSP. This has been demonstrated by use of site-directed mutagenesis to replace conserved threonine residues in proteins such as nicotinic acetylcholine receptor, fungal nitric oxide reductase (cytochrome P450nor), Staphylococcal enterotoxin B, and type I antifreeze polypeptide from flounder (*41−44*). The authors of these studies have proposed an involvement of Thr hydroxyl and methyl groups in hydrogen and hydrophobic bonding interactions,

respectively. In light of these related findings, it seems probable that Thr 7 and 15 are required to target MSP to specific docking sites on PSII, and that the interactions between these residues and sites on PSII consist of hydrogen bonding or hydrophobic interactions. Further investigations of the possible roles of Thr7 and Thr15 are currently under way.

An inspection of the near-UV CD spectra shown in Figure 4 shows that mutants ΔR5M, ΔL6M, and ΔT15M possess a domain where aromatic amino acid residues are in a more hydrophobic environment than they are in wild-type protein, or in mutant ΔT7M, as evidenced by the greater intensity of Tyr (285 nm) and Trp (292 nm) peaks in these spectra. The lone Trp in MSP is located at the position 241 in a hydrophobic region in the C-terminus of the protein (*45−47*), so changes in the near-UV CD features of Trp caused by N-terminal truncations may indicate the presence of interactions between the N- and C-termini of MSP, as proposed by Enami et al. (*48*) on the basis of cross-linking experiments. Because Tyr residues are distributed throughout the entire MSP sequence, we cannot determine whether changes in its spectral features originate from all tyrosines or only from Tyr242 in the hydrophobic domain. For the case of Trp, and perhaps Tyr 242 as well, near-UV CD shows that deletions of amino acid residues at the MSP N-terminus causes changes in the tertiary structure of the mutant proteins that affect the hydrophobic environment of these aromatic residues.

In parallel with these changes, the ΔR5M, ΔL6M, and ΔT15M mutants exhibited an ability to bind to nonspecific sites on PSII (compare Figure 4 with Figure 1 and Table 1). This same behavior was also observed with the mutants characterized in refs *34* and *35*. A comparison of the third and fifth columns in Table 2 shows that the N-terminal deletion mutants of MSP, where the hydrophobic environment surrounding Tyr or Trp residues either increased or decreased in comparison to the wild-type protein, were also capable of some level of nonspecific binding to usw- and intact PSII. About 1.5 mol of ΔG3M, 1 mol of ΔS13M or ΔK14M, and 0.4 mol of ΔE18M were bound when 10 mol of MSP/mol of intact PSII was present in the reconstitution mixture; for the other mutants see Table 1. In light of these results, we have reexamined the binding behavior of mutant ΔE10M. When ΔE10M binding to usw-PSII was carried out, a gradually increasing, nonsaturating binding curve was

obtained. While 1.3 mol of ΔE10M/mol of PSII was bound at 2 mol of ΔE10M/mol of PSII, almost 4 mol of ΔE10M/mol of PSII was bound at 10 mol of ΔE10M/mol of PSII used for reconstitution (data not shown). Experiments with intact PSII showed maximal nonspecific binding of almost 1 mol of ΔE10M/mol of intact PSII after incubation of 10 mol of ΔE10M/mol of intact PSII (see Table 1). In contrast to the mutants described above, wild-type MSP and the ΔT7M mutant have tertiary structures that prevent nonspecific binding of MSP to PSII (see the third and fifth columns in Table 2). In light of these considerations, it is apparent that although they may have no direct role in the functional binding of MSP to PSII, the N-terminal sequences [1]-EGGKR[6]L, [8]YDEIQS[14]K, and [16]YL[18]E fulfill an essential role in maintaining the tertiary solution structure that ensures highly specific functional binding of MSP to PSII. The presence of these sequences in MSP makes it possible for the protein tertiary structure to be incompatible with nonspecific binding sites on PSII. Deletion of amino acids from these three sequences, on the other hand, produces a solution structure that does not interfere with specific binding of MSP to PSII, but which does permit nonspecific binding of the protein to PSII. Nonspecific binding of MSP to PSII induced by mutagenesis is not unique to N-terminal truncations of MSP. A similar type of binding was reported by Motoki et al. (49), who examined the effects of mutations in the sequence [148]V-[163]G of MSP from *Synechococcus elongatus*. Alterations of [152]Arg, [158]Asp, [160]Lys, and [162]Arg induced significant nonspecific binding of MSP to PSII.

The secondary structure of MSP appears to be less sensitive to N-terminal truncations than is its tertiary structure because no changes in the MSP secondary structure were observed until a substantial deletion (18 amino acids in the ΔE18M mutant) was carried out. This deletion generated a small increase in the content of unordered structures at the expense of β-sheets (see ref 35). Although our result might support some studies (7, 50, 51), predicting β-sheet around the [18]E amino acid residue, there is no evidence in our secondary structure analyses that would indicate a loss of predicted secondary structure elements upon deletion of residues within the [1]E-[15]T MSP sequence. Until there are improvements in the protein databases (additions of more unfolded proteins) that are employed for far-UV CD spectral analyses, it will be difficult to determine if other, subtle changes have occurred in the secondary structure contents of the mutants that have been used in the studies presented here and in refs 34 and 35.

In conclusion, results presented in this study and in refs 34 and 35 provide new information on the molecular basis of the highly specific interaction between MSP and PSII. First, threonines 7 and 15, the first amino acid residues in the highly conserved motifs T(Y/F)DE and TY in the eukaryotic MSP sequence, are essential for functional binding of two copies of MSP to PSII. The crystal structures of cyanobacterial PSII (52, 53) are interpreted as showing that the prokaryotic enzyme contains one copy of MSP. A possible explanation of differences in MSP content in eukaryotes and prokaryotes is proposed in ref 35, which showed that cyanobacteria lack one of the two TY motifs present at the N-terminus of eukaryotic MSP. This hypothesis is further refined by the data reported here on the roles of two Thr residues in binding two copies of MSP to spinach PSII. We conclude that Thr7 and Thr15 may target MSP to specific docking sites on PSII, and/or may form hydrogen or hydrophobic bonds between MSP and PSII. Second, on the basis of protein solution structure analysis combined with reconstitution experiments, the presence of the N-terminal domains [1]EGGKR[6]L, [8]YDEIQS[14]K, and [16]YL[18]E regulate MSP's tertiary structure to optimize specific binding to PSII and to prevent nonspecific binding interactions. Removal of these domains modifies MSP tertiary structure so that nonspecific binding to PSII can occur after native, high affinity MSP binding sites on the enzyme have been occupied.

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