N-Terminus of the Photosystem II Manganese Stabilizing Protein: Effects of Sequence Elongation and Truncation[†]

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Received October 2, 2001; Revised Manuscript Received December 27, 2001

ABSTRACT: The importance of the N-terminal domain of manganese stabilizing protein in binding to photosystem II has been previously demonstrated [Eaton-Rye and Murata (1989) *Biochim. Biophys. Acta 977*, 219−226; Odom and Bricker (1992) *Biochemistry 31*, 5616−5620]. In this paper, we report results from a systematic study of functional and structural consequences of N-terminal elongation and truncation of manganese stabilizing protein. Precursor manganese stabilizing protein is the unprocessed wild-type protein, which carries an N-terminal extension of 84 amino acids in the form of its chloroplastic signal peptide. Despite its increased size, this protein is able to reconstitute O_2 evolution activity to levels observed with the mature, processed protein, but it also binds nonspecifically to PSII. Truncation of wild-type manganese stabilizing protein by site-directed mutagenesis to remove three N-terminal amino acids, resulting in a mutant called ΔG3M, causes no loss of activity reconstitution, but this protein also exhibits nonspecific binding. Further truncation of the wild-type protein by ten N-terminal amino acids, producing ΔE10M, limits binding of manganese stabilizing protein to 1 mol/mol of photosystem II and decreases activity reconstitution to about 65% of that obtained with the wild-type protein. Because two copies of wild type normally bind to photosystem II, amino acids in the domain ^{4}K−^{10}E must be involved in the binding of one copy of manganese stabilizing protein to photosystem II. Spectroscopic analysis (CD and UV spectra) reveals that N-terminal elongation and deletion of manganese stabilizing protein influence its overall conformation, even though secondary structure content is not perturbed. Our data suggest that the solution structure of manganese stabilizing protein attains a more compact solution structure upon removal of N-terminal amino acids.

Oxidation of water by photosystem II (PSII)[1] is the source of molecular O_2, electrons, and protons in higher plants, algae, and cyanobacteria. Photosystem II is a multisubunit pigment−protein complex localized in thylakoid membranes of chloroplasts that contains intrinsic and extrinsic components. Seven major intrinsic proteins, CP47, CP43, D1, D2, α and β subunits of cytochrome *b*-559, and the *psb*I gene product form the membrane-associated core of PSII. Three extrinsic proteins of 17, 23, and 33 kDa occupy the lumenal domain of PSII and form part of what is called the O_2 evolving complex (OEC). The smaller proteins (17 and 23 kDa) facilitate retention of inorganic cofactors (Ca^{2+} and Cl^{-}) by PSII (*1*, *2*). The largest protein (33 kDa), called manganese stabilizing protein, or MSP, stabilizes the tetranuclear manganese cluster under physiological salt conditions (*3*−*5*) and accelerates O_2 evolution activity (*6*, *7*).

Manganese stabilizing protein is a thermostable, natively unfolded polypeptide (*8*). It recovers its solution secondary structure and functional properties after incubation for 2 h at 90 °C and also exhibits unusual behavior in solution, as detected by gel filtration and SDS−PAGE. Although its molecular mass from DNA sequencing is 26540 Da (*9*), MSP migrates at about 33 kDa on SDS−PAGE (*10*) and has an estimated mass of about 38−40 kDa based on gel filtration experiments (*8*, *11*). Like other natively unfolded proteins, MSP is an acidic protein [p*I* = 5.2 (*10*)]. Secondary structure prediction, based on far-UV CD spectra, indicates the presence of about 7−9% α-helix, 33−44% β-sheet, and 49−58% turns and random coil (*12*−*14*). The C-terminal tripeptide, LEQ, of MSP is required for its quantitative assembly into PSII and for high rates of O_2 evolution (*11*);

[†] This research was supported by a grant to C.F.Y. from the National Science Foundation (MCB-0110455).

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[1] Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CD, circular dichroism; Chl, chlorophyll; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; IPTG, isopropyl β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese stabilizing protein; OEC, O_2 evolving complex; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pET, plasmid for gene expression with T7 RNA polymerase; PS, photosystem; *psb*O, gene encoding precursor MSP; SDS, sodium dodecyl sulfate; sw-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic proteins; Tris, tris(hydroxymethyl)-aminomethane; unrd, unordered structure; 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.

10.1021/bi0118761 CCC: $22.00
Published on Web 01/31/2002

Leu 245 is the essential residue of the tripeptide that is necessary to maintain MSP in a conformation that promotes efficient, fully functional binding to PSII (*14*).

Estimates, by biochemical approaches, of the amount of MSP bound to PSII indicate that 2 mol are bound per mole of PSII reaction center. This stoichiometry has been obtained by direct quantification of the amount of MSP bound to unperturbed PSII membranes (*15*, *16*) and by determinations of a requirement for rebinding of 2 mol of the protein to MSP-depleted PSII for full restoration of activity (*17*). Binding of both native (*18*) and modified forms (*19*, *20*) of MSP exhibits cooperative binding behaviors, as might be expected if more than one copy of the protein were bound per reaction center, and a temperature-sensitive MSP mutant (V235A) affects PSII binding of one, but not both, copies of MSP (*21*). Results from structural investigations are not as uniform. Cryoelectron microscopy has yielded constructed images of PSII supercomplexes at ca. 24 Å resolution, and the authors assign a globular projection on the lumenal surface of these images to a single copy of MSP (*22*). Transmission electron microscopy, using analyses of images from negatively stained PSII preparations, intact or polypeptide depleted, concluded that one or two copies of MSP, depending on the shape of the protein, could be accommodated in the globular mass assigned to the subunit (*23*). The 3.8 Å structure of the PSII reaction center of *Synechococcus elongatus* is interpreted to show a single copy of MSP (*24*), which exhibits a cylindrical tertiary conformation comprised of β-sheet domains, rather than the globular structure constructed from cryoelectron microscopy (*22*).

Several studies demonstrated binding of MSP to CP47 (*25*−*27*), and biotinylation and mutagenesis studies subsequently identified the E loop of CP47 as one site of MSP binding (*28*, *29*). Enami et al. (*30*) have proposed additional binding sites for MSP associated with CP43 and cytochrome *b*-559. Carboxyl groups of aspartyl and glutamyl residues (D and E) and amino groups of lysine and arginine residues (K and R) have been proposed to play a role in electrostatic interactions between MSP and PSII (*19*, *26*, *28*, *31*). Odom and Bricker (*32*) used cross-linking by EDC to show that MSP and PSII interact through domains ^{1}E−^{76}K of MSP and ^{364}E−^{440}D of the large extrinsic E loop of CP47. For CP47, this observation was further confirmed by site-directed mutagenesis of the E loop in *Synechocystis* 6803. Although mutagenesis of certain conserved, charged residues (^{358}R, ^{359}R, and ^{364}E) of CP47 showed that they are not required for PSII function (*33*), other experiments revealed that alterations in the ability to evolve O_2 occurred when mutations were introduced into ^{384}R and ^{385}R of the CP47 E loop (*34*, *35*). Positive charges on these residues are proposed to be necessary for O_2 evolution, perhaps because they may play a role in the interaction between CP47 and MSP (*35*). The MSP N-terminus was implicated in binding to PSII by Eaton-Rye and Murata (*36*), who removed the first 16 or 18 amino acid residues from the protein's N-terminus using chymotrypsin and *Staphylococcus aureus* strain V8 protease, respectively. These truncated MSP species were incapable of binding to PSII (*36*). Site-directed mutagenesis showed that the highly conserved aspartate residue at position 9 of MSP was not essential for its interaction with PSII; mutation of spinach MSP, replacing ^{9}D by K (D9K) and N (D9N), respectively, produced mutants that exhibit O_2 evolution activity at levels observed with the wild-type protein (*37*). In *Synechocystis* sp. PCC6803, the N-terminal MSP mutation D9K accumulated to normal levels and exhibited only slightly altered activity, relative to the wild-type strain (*38*).

In the work reported here, we have combined site-directed mutagenesis and overexpression of MSP in *Escherichia coli* with biochemical and spectroscopic methods to examine more systematically the role of MSP's N-terminus in binding to PSII and in restoration of O_2 evolution activity. Effects of protein elongation and truncation were explored. Results of these experiments showed that elongation of MSP by retention of the transit peptide sequence (precursor MSP, or preMSP) and truncation by three N-terminal amino acid residues have little, if any, effect on the biological function of MSP. With regard to their binding interactions with PSII, both proteins exhibited a higher, nonspecific, level of binding to PSII than did the mature wild-type protein. A more extensive truncation, by ten N-terminal amino acid residues, reduced MSP binding to PSII from two to one copy per reaction center and produced a partial restoration of O_2 evolution activity (65% of that obtained with wild-type protein). We also report results of experiments focused on the characterization of protein structure (size-exclusion chromatography and CD and UV spectroscopy) that show that N-terminal elongation and deletion of MSP do not influence secondary structure. Changes in solution conformation are detected, however; MSP becomes more compact upon removal of N-terminal amino acids.

MATERIALS AND METHODS

PSII Membranes: Preparation and Extraction of Extrinsic Polypeptides. Photosystem II membranes were isolated from fresh market spinach according to Berthold et al. (*39*) with modifications described in ref *40*. Extrinsic proteins were extracted sequentially. The 17 and 23 kDa polypeptides were extracted by incubation of PSII in 2 M NaCl, 1 mM EDTA, and 50 mM MES (pH 6), which yielded sw-PSII. Next, MSP was extracted by incubation of sw-PSII in the dark in 50 mM MES (pH 6), 3.1 M urea, and 240 mM NaCl on ice for 30 min to produce usw-PSII. The resulting usw-PSII membranes were washed once in SMN-200 buffer [0.4 M sucrose, 50 mM MES (pH 6), and 200 mM NaCl] before they were suspended in SMN-200 and stored at −70 °C.

Reconstitution of PSII with MSP and Functional Analysis. Reconstitution mixtures were prepared as described in ref *41* except that 100 mM NaCl and 20% betaine (w/v) were present instead of 60 mM NaCl and 2% betaine (w/v). The O_2 evolution activities of PSII samples were assayed as described in ref *41*. Typical sample activities were as follows: intact PSII, 500−600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}; sw-PSII, 300−450 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}; usw-PSII, about 200 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. The extent of rebinding MSP to PSII was determined by SDS−polyacrylamide gel electrophoresis (*41*). The relative amount of MSP bound to PSII was estimated by integration of Coomassie-stained MSP bands using Sigmagel software (Jandel Scientific). The MSP content of a salt-washed PSII sample was taken as the control (100% MSP bound), except for the experiments using intact PSII to detect nonspecific protein binding (Table 4). In these cases, Coomassie-stained MSP (and CP43 in Table 4) bands on SDS gels were taken

as the control, natively bound level of MSP and the concentration of CP43, respectively. Staining intensities were normalized to the integral of the Coomassie-stained 47 kDa band to correct for small errors in loading of protein samples to the gel. The MSP rebinding and activity reconstitution data presented in the figures are the averages of three separate sets of experiments.

Mutations of psbO cDNA and Transformations of E. coli Cells. For construction of DNA encoding recombinant wild type and preMSP, the oligonucleotides 5′-CCATGGATGGCAGCTTCATTACAAGCATC-3′ and 5′-TGAATTCCTTTTATTGCTCAAGTTGTGC-3′ were designed and synthesized (Life Technology, Gibco BRL custom primers) on the basis of the sense strand of the N-terminal and antisense strand of the C-terminal encoding region of MSP, respectively. For ΔG3M and ΔE10M mutations, the oligonucleotides 5′-ACATATGAAGAGATTGACCTACGAC-3′ and 5′-ACATATGA TCCAGAGCAAGACATAC-3′, respectively, were designed and synthesized on the basis of the sense strand of the N-terminal encoding region of MSP. The same oligonucleotide was used for the 3′ primer in all cases. Oligonucleotides encoding N-terminal amino acid sequences contain either a *Nco*I (for wild type) or *Nde*I (for deletion mutants) endonuclease restriction site, and the C-terminal oligonucleotide contains an *Eco*RI endonuclease restriction site, three nucleotides after the stop codon of *psb*O. The coding region was amplified by polymerase chain reaction (PCR) using the oligonucleotides described above as primers. PCR products were ligated into the pCR T7/CT TOPO vector, and the cloned reaction products were transformed into TOP 10 F′ (Invitrogen) cells. After confirmation of the insert by sequencing, the mutant *psb*O genes were subcloned into pET8c (for wild type) or pET11a (for deletion mutants) translation vectors, respectively (*42*). The pET *psb*O construct was then inserted into BL21(DE3)pLysS *E. coli* cells. All mutations were confirmed by DNA sequencing. The integrity of N-terminal recombinant samples was also confirmed by amino acid sequencing (Edman degradation). The expected sequences (AASLQ for preMSP, EGGKR for wild-type MSP, MKRLT for ΔG3M, and MIQSK for ΔE10M) were found.

Overexpression and Purification of Recombinant MSP. All recombinant proteins were overexpressed in *E. coli*, and inclusion bodies were isolated as described in ref *42* with some modifications: 50 μg/mL ampicillin was added to LB media for overexpression of ΔG3M and ΔE10M, and higher concentrations of IPTG (0.4−1 mM) were used for overexpression of preMSP and the truncation mutants. Wild-type MSP, ΔG3M, and ΔE10M were extracted from inclusion bodies by incubation in solubilization buffer [3 M urea, 10% betaine (w/v), 20 mM Bis-Tris (pH 6.4), 10 mM NaCl] for 2 h at room temperature. Each solubilized recombinant protein was loaded onto a Pharmacia Resource Q column equilibrated with solubilization buffer. A linear gradient (5−250 mM NaCl) was applied and recombinant MSP eluted between 120 and 150 mM NaCl. The sample was diluted about 9-fold and applied to the same FPLC column equilibrated with solubilization buffer. A step gradient (30 mM, 150 mM, 500 mM, and 1 M NaCl) was used as the second chromatographic step to further purify MSP, which was eluted at 150 mM NaCl. Urea was next removed from the recombinant protein solution by dialysis against 100 mM Tris (pH 8) and 10 mM NaCl, and the pH was adjusted by dialysis in 50 mM MES (pH 6) and 10 mM NaCl. For preMSP, inclusion bodies were treated in two steps. First, solubilization [in 2.6 M urea, 5% betaine (w/v), 20 mM Bis-Tris (pH 6.4), 5 mM NaCl] and centrifugation (48000*g*, 4 °C, 30 min) released mature MSP into the supernatant. Second, the resulting pellet was solubilized in a medium containing a higher urea concentration [6 M urea, 10% betaine (w/v), 20 mM Bis-Tris (pH 6.4), 10 mM NaCl]. After 2 h incubation at room temperature and centrifugation (48000*g*, 4 °C, 30 min), the protein in the supernatant was loaded onto a 3 M urea-equilibrated Resource Q column. After elution of preMSP from the column as described above for the truncation mutants, dialysis against Tris and MES buffers (see above) was employed. Protein concentrations were estimated spectrophotometrically at 276 nm using an extinction coefficient of 16 mM^{-1} cm^{-1} (*16*). Sucrose (0.4 M) was added before proteins were stored at −70 °C.

CD and UV Spectroscopy. Samples of MSP that were used for CD and UV spectra were dialyzed against 10 mM KH_2PO_4 buffer, pH 6, and diluted to a concentration of 10 μM. The CD spectra were obtained using an AVIV 62 DS CD spectrometer calibrated with (+)10-camphorsulfonic acid (1 mg/mL in H_2O) (*43*). Experimental conditions for far-UV and near-UV CD spectral measurements are shown in the figure legends. Far-UV CD spectra were analyzed using CONTIN/LL and CDSSTR methods from the CDPro package downloaded from the Internet (http://lamar.colostate.edu/~sreeram/CDPro). Detailed information about methods and basis sets can be found in ref *44*. On the basis of the amino acid sequence of MSP, secondary structure prediction was obtained from the PSIPRED V 2.0 protein structure prediction server located at the web site http://insulin.brunel.ac.uk/psipred (for more information, see refs *45*−*48*). UV spectra were collected on an OLIS-modified Cary-17 instrument. Experimental conditions are presented in the legend to Figure 8.

Size-Exclusion Chromatography. For size-exclusion chromatography, a Superose-12 column on a Pharmacia FPLC system was used (*14*). A calibration curve was constructed using the standards described in ref *14*. The column was equilibrated under nondenaturing conditions with 20 mM Bis-Tris (pH 6.4) and 150 mM NaCl buffer that was degassed using a vacuum pump and sonic bath. For denaturing conditions, 3 or 6 M urea, respectively, was added to the buffer system. The values of apparent molecular masses obtained by this method diverged from one another at most by ±6%.

RESULTS

MSP Elongation: Functional Characterization of PreMSP. The N-terminal amino acid sequence of precursor MSP (preMSP), shown in abbreviated form in Table 1, is an 84 amino acid transit peptide, which increases its calculated molecular mass from 26.5 to 35 kDa. The protein can be overexpressed in *E. coli*: The ratio of accumulation of preMSP to mature MSP can be increased by changing the conditions of overexpression (*42*), specifically by inducing protein expression with high concentrations of IPTG. To determine how extending the N-terminus of MSP affects its ability to bind and reconstitute function to the OEC, preMSP

Table 1: N-Terminal Amino Acid Sequences of Wild-Type, Precursor, and Deletion Mutants of Recombinant MSP of Spinach[a]

Protein	N-terminal sequence
Wildtype	**E G G K R** L T Y **D E** I Q S **K** T
PreMSP	M A A S L Q ... **E G G K R** L T Y **D E** I Q S **K** T
ΔG3M	M - - - - - - - - - - - -
ΔE10M	M - - - - -

[a] Dashed lines indicate conserved amino acid residues in all sequences; dots (...) represent the remaining 78 amino acid residues of the transit peptide sequence; positively and negatively charged residues are shown in boldface type.

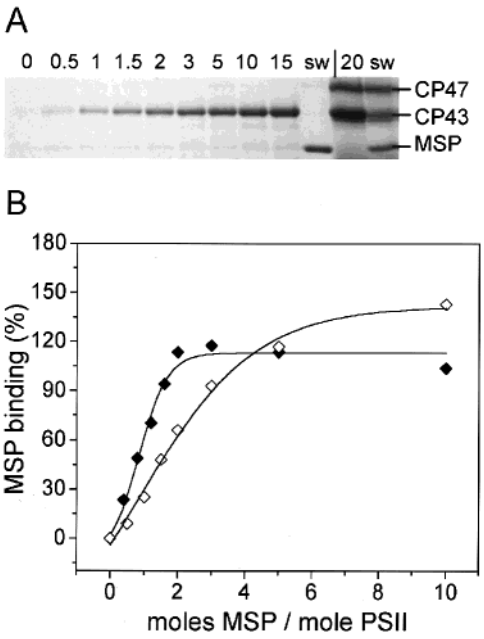

FIGURE 1: (A) Binding of preMSP to PSII. A Coomassie blue stained SDS−PAGE gel of usw-PSII samples reconstituted with precursor manganese stabilizing protein (preMSP) for 1 h at 22 °C is shown. Because preMSP comigrates with CP43, PSII-bound preMSP was solubilized by incubation of reconstituted samples in 3 M urea. Supernatant fractions (first 10 gel lanes) were then analyzed by SDS−PAGE. The moles of MSP per mole of PSII used for reconstitution is indicated at the top of the lanes. sw, control sw-PSII; lane 20, PSII reconstituted with 20 mol of preMSP/mol of PSII. (B) Binding curve for usw-PSII reconstituted with mature and precursor wild-type MSP, respectively: solid symbols, mature wild type; open symbols, preMSP. SDS−PAGE gels of reconstituted samples were analyzed by densitometry, and binding efficiencies of wild-type and precursor MSP are expressed as the percentage of MSP in sw-PSII (100% control).

was used to reconstitute urea-washed PSII membranes (see Materials and Methods). Figure 1A presents a gel showing rebinding of preMSP to PSII. Unbound or weakly bound protein was removed by centrifugation and washing of PSII membrane pellets. Because preMSP comigrated with CP43 on SDS gels, bound preMSP was released from reconstituted PSII membranes using 3 M urea and 200 mM NaCl and then electrophoresed on a SDS−PAGE gel. Salt-washed PSII membranes, containing native MSP, were used as control samples. Quantitative evaluation of an SDS gel is shown in Figure 1B, and mature wild-type binding is presented for comparison (for the corresponding gel, see Figure 1A, top, in ref *21*). As can be seen, the amount of preMSP rebound

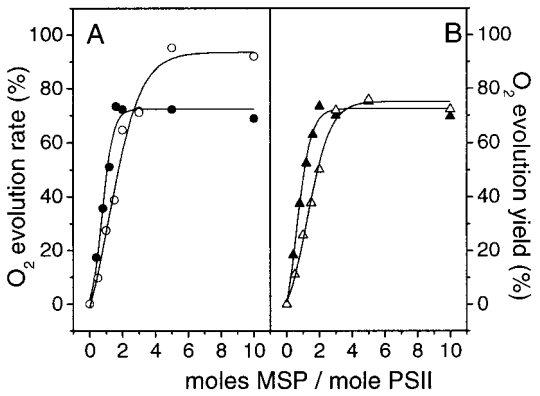

FIGURE 2: Recovery of O_2 evolution activity by usw-PSII membranes reconstituted with mature and precursor wild-type MSP, respectively. (A) O_2 rate and (B) O_2 yield assayed during 1 and 4 min of continuous illumination, respectively. Samples were assayed after reconstitution incubation at 22 °C for 1 h. Residual activity of usw-PSII was subtracted from the activities of reconstituted samples. 100% corresponds to the activity of control sw-PSII. Solid symbols, mature wild type (redrawn from ref *21*); open symbols, preMSP.

to PSII was the same as that of mature wild-type MSP when about 4 mol of preMSP was added to PSII during reconstitution. Binding did not saturate, however, and attained a level of binding estimated to be about 140% of the sw-PSII control value when usw-PSII was incubated with 10 mol of preMSP/mol of PSII. For comparison, PSII binding of the mature wild-type protein saturates at about 2 mol of MSP/mol of PSII and remains fully saturated at 10 mol of MSP/mol of PSII.

The activity assays presented in Figure 2 show that preMSP was fully effective in restoration of O_2 evolution activity. While the 4 min O_2 yield was comparable with that of recombinant mature wild-type protein, the O_2 evolution rate activity was somewhat higher, about 90% of the control, salt-washed value after incubation of usw-PSII with 10 mol of preMSP/mol of PSII. These data clearly indicate that although N-terminal elongation of mature wild-type MSP by its transit peptide affects the protein's binding affinity, it does not interfere with the function of bound preMSP. This result is in agreement with the results of Seidler, who found that a six-His extension of the N-terminus did not affect MSP binding to PSII (*20*).

MSP Truncations: Functional Characterization of ΔG3M and ΔE10M. To systematically examine the role of the N-terminus of MSP, truncations were also prepared by substituting translation initiation codons (Met residues) at two positions in the N-terminal sequence of MSP; the resulting sequences are given in Table 1. The first truncation is in theory conservative and deleted only the first three amino acid residues, EGG, to produce a recombinant mutant protein, ΔG3M. The effects of this mutation on rebinding and activity reconstitution assays are shown in Figures 3 and 4. The SDS−PAGE gel shows that the mutant MSP migrated slightly faster than wild type. This behavior is consistent with a modest reduction in size and/or charge of the mutant protein. The ΔG3M mutation had a substantial effect on the apparent stoichiometry of MSP binding (Figure 3A). Quantitative evaluation (Figure 3C) determined that the binding of ΔG3M was normal and very similar to that of wild type up to the point where 2 mol of MSP/mol of PSII had bound

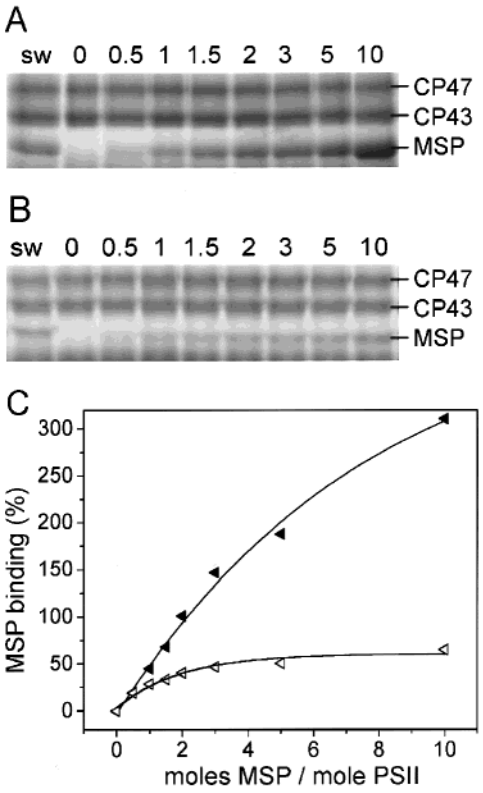

FIGURE 3: Coomassie blue stained SDS−PAGE gel of usw-PSII samples reconstituted with ΔG3M (A) and ΔE10M (B), respectively, for 1 h at room temperature. The moles of MSP per mole of PSII used for reconstitution is indicated at the top of the lanes. sw, control sw-PSII. (C) Binding curves for usw-PSII reconstituted with ΔG3M and ΔE10M, respectively: solid symbols, ΔG3M; open symbols, ΔE10M. SDS−PAGE gels of reconstituted samples were analyzed by densitometry, and the binding efficiency of ΔG3M and ΔE10M was expressed as the percentage of MSP in sw-PSII (100% control); Coomassie staining of CP47 was used as an internal standard to control for slight differences in protein loading.

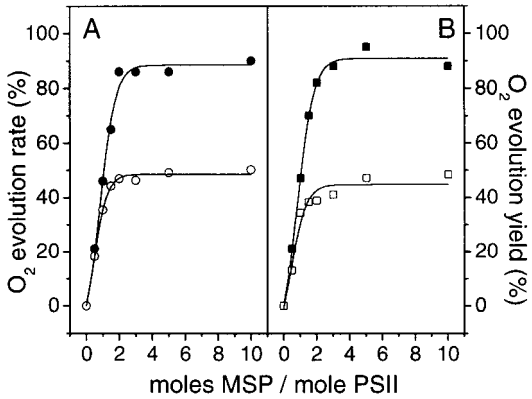

FIGURE 4: Reconstitution of O_2 evolution activity of usw-PSII membranes by ΔG3M and ΔE10M, respectively. (A) O_2 rate and (B) O_2 yield assayed for 1 and 4 min of continuous illumination, respectively. Solid symbols, ΔG3M; open symbols, ΔE10M. Samples were assayed after reconstitution incubation (for 1 h) at room temperature. Determination of 0% and 100% values is given in the legend of Figure 2.

(compare Figures 1B and 3C). Upon addition of higher protein concentrations to reconstitution mixtures, however, ΔG3M binding increased and did not attain saturation at the highest protein concentrations used in these reconstitution experiments. At this protein concentration (10 mol/mol of PSII), about 6 mol of ΔG3M/mol of PSII was bound; correcting for specific binding of 2 mol of ΔG3M indicates that about 4 mol of this MSP mutant binds nonspecifically to PSII under these conditions. Despite this clear evidence for substantial nonspecific binding, the shapes of activity restoration curves (Figure 4) are very similar to those observed with wild-type recombinant protein (Figure 2), with activity reaching maximum levels at about 2 mol of ΔG3M/mol of PSII. However, at saturation, the rate of O_2 evolution activity was higher than wild type by about 20%.

Table 2: Apparent Molecular Masses of Wild-Type, Precursor, and Deletion Mutants of MSP Based on Size-Exclusion Chromatography

protein	apparent molecular mass (kDa)	no. of peaks	apparent molecular mass (kDa) in 3 M urea	apparent molecular mass (kDa) in 6 M urea
wild type	37	1	ND[a]	ND
preMSP	49/91	2	114/184	138/244
ΔG3M	34	1	ND	ND
ΔE10M	33	1	ND	ND

[a] ND, not determined.

A second truncation mutation of spinach MSP was prepared, which deleted ten N-terminal amino acid residues, EGGKRLTYDE, and resulted in the recombinant protein ΔE10M (Table 1). Results of reconstitution analyses with this protein revealed a negative effect of the deletion on MSP rebinding to PSII and on recovery of O_2 evolution activity as well. On a SDS−PAGE gel, ΔE10M migrated faster than wild type (Figure 3B), probably for the same reasons as given for ΔG3M. Rebinding assays showed that ΔE10M can rebind to PSII, but the level of reconstitution at saturation resulted in only 50% MSP rebinding as compared to the MSP contents of a salt-washed control (1 mol of ΔE10M/mol of PSII was bound; Figure 3C). It therefore seems likely that only one copy of this MSP truncation mutant can bind to PSII membranes. This binding also occurs with a lowered affinity; a higher stoichiometry of added protein (3 mol of ΔE10M/mol of PSII) was required to achieve binding saturation. This indicates a defect in the ability of ΔE10M either to assemble with PSII membranes or to remain bound once assembly has occurred. Relative to wild-type MSP, restoration of O_2 evolution activity of ΔE10M was impaired less than its rebinding (Figure 4). When reconstituted with between 2−3 mol of MSP/mol of PSII, O_2 evolution rates and yields (panels A and B of Figure 4, respectively) attained saturation, but only 45% of the control activity of a sw-PSII preparation was recovered under these conditions. Comparing this result to those obtained in reconstitution experiments with recombinant wild-type MSP, restoration of O_2 evolution activity by ΔE10M was lowered by about 25%.

N-Terminal Elongation and Truncation of MSP Influence Its Solution Conformation. To investigate the types of structural changes that could be connected with functional characteristics of the MSP species just described, size-exclusion chromatography and CD and UV spectroscopy were employed. As a natively unfolded protein (*8*), MSP exhibits a higher apparent molecular mass on size-exclusion chromatography relative to the molecular mass calculated from its DNA sequence derived amino acid sequence (*8*, *9*, *11*). Data from size-exclusion chromatography experiments are presented in Table 2 and reveal two interesting results: First, preMSP was eluted as two peaks, corresponding to molecular masses of 49.3 and 90.9 kDa. The approximately

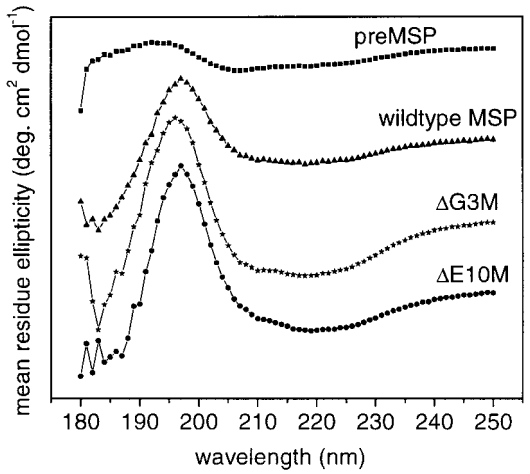

FIGURE 5: Far-UV CD spectra (protein solution minus buffer) of precursor, wild type, and N-terminal deletion mutants of MSP. The protein concentration was 10 μM in 10 mM KH_2PO_4 buffer, pH 6.0. Each spectrum is the average of 20 scans. Experimental conditions were as follows: scan width, 250−180 nm; time constant, 1 s; bandwidth, 1 nm; temperature, 25 °C; path length, 1 mm; sample volume, 300 μL.

2-fold difference in masses of these peaks indicates that preMSP forms monomers and dimers. Formation of preMSP dimers results from strong interactions because neither 3 nor 6 M urea could dissociate them (see Table 2). There are two Cys residues located in the transit peptide of MSP that might form −S−S− bonds that would form a stable dimer of preMSP. To test for the existence of disulfide bonds, 6 mM DTT was added to 6 M urea to eliminate these bonds during gel filtration chromatography, but no effect was observed (data not shown). A role of disulfide bonds in dimer formation cannot be excluded, however, on the basis of the results presented in ref *49*, where it was shown that dissociation of dimeric plastocyanin into monomers on SDS−PAGE required 10% mercaptoethanol. Thus, it is possible that DTT concentrations used in our experiments were not sufficiently high to reduce disulfide bonds between preMSP monomers. A second significant observation in Table 2 is that both of the deletion mutants, ΔG3M and ΔE10M, exhibit lower molecular masses than recombinant wild-type MSP; ΔG3M is estimated to be 34 kDa, and ΔE10M is 33 kDa.

Using far-UV CD spectroscopy, the results presented in Figure 5 were obtained. The order of protein CD spectra in Figure 5 from top to bottom follows the gradual reduction in the number of N-terminal amino acid residues, starting with preMSP. As can be seen, the amplitude of mean residue ellipticity is highest for the shortest MSP (compare Figure 5 and Table 1) and decreases with the increasing number of residues at the N-terminus. Far-UV CD spectra provide information about the secondary structure of proteins. Analysis of the CD spectra in Figure 5, using CONTIN/LL and CDSSTR methods (see Materials and Methods) and different protein reference sets, provided the secondary structure predictions presented in Table 3. Secondary structure prediction using basis sets 6 and 7 only (for analysis of MSP CD spectra) is proposed to be more accurate for MSP because these two sets contain CD spectra of five unfolded proteins (*44*). Data in Table 3 clearly demonstrate that secondary structure contents of the MSP species are very

Table 3: Secondary Structure Prediction for Wild-Type, Precursor, and Deletion Mutants of MSP Based on the Far-UV CD Spectral Analysis

	basis sets 1, 3, 4, 6, and 7[a]				basis sets 6 and 7 only			
protein	α-helix	β-sheet	turn + unrd	total	α-helix	β-sheet	turn + unrd	total
wild type	4	40	55	99	3	41	56	100
preMSP	5	42	52	99	5	37	57	99
ΔG3M	6	37	56	99	4	37	58	99
ΔE10M	6	39	55	100	4	37	59	100

[a] For detailed basis description see ref *44*; numbers are averages of results obtained from given basis sets.

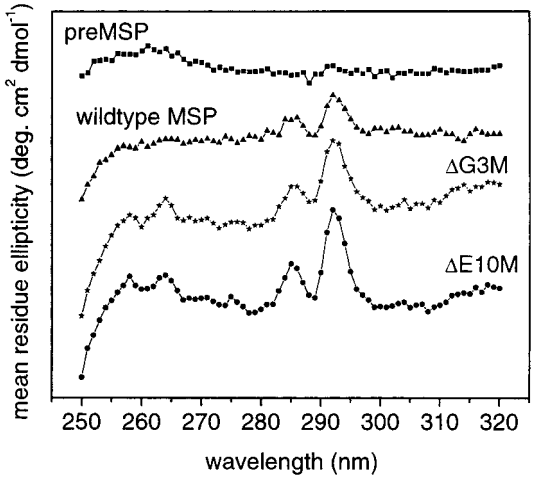

FIGURE 6: Near-UV CD spectra (protein solution minus buffer) of precursor, wild type, and N-terminal deletion mutants of MSP. Experimental conditions were as follows: scan width, 320−250 nm; path length, 1 cm; sample volume, 1 mL. Other experimental conditions are as in Figure 5.

similar. These results indicate that (i) the estimated secondary structure content of these proteins is not reflected in the amplitude of mean residue ellipticity in their far-UV CD spectra and (ii) the N-terminal elongation and deletions of MSP have no significant effect on the estimated secondary structure of protein as monitored by CD spectroscopy. These results are in strong contrast to the results obtained for C-terminal truncation mutations, where loss of β-sheet structure was replaced by random coil and other features (*14*).

Near-UV CD spectroscopy can provide information on the tertiary structure of proteins (*50*). Characteristic peaks of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) in near-UV CD spectra depend on their environment. Changes in tertiary structure of proteins, which affect the environment of these amino acid residues, will be mirrored in the amplitude of their CD spectral features. Figure 6 depicts near-UV CD spectra of precursor, wild type, and deletion mutants of MSP. While the wild-type MSP spectrum reveals peaks at 285 nm (assigned to tyrosine) and 292 nm (assigned to tryptophan) (*50*), the spectrum of preMSP has almost undistinguishable peaks at these wavelengths, whereas the spectrum of ΔG3M contains tyrosine and tryptophan peaks of higher intensity, compared to wild type. Moreover, an additional small peak at 264 nm appears in the near-UV CD spectrum of ΔG3M, which might arise from phenylalanine (*50*). The changes observed in the ΔG3M spectrum were further emphasized in the near-UV CD spectrum of ΔE10M. The relationship between the intensity of amino

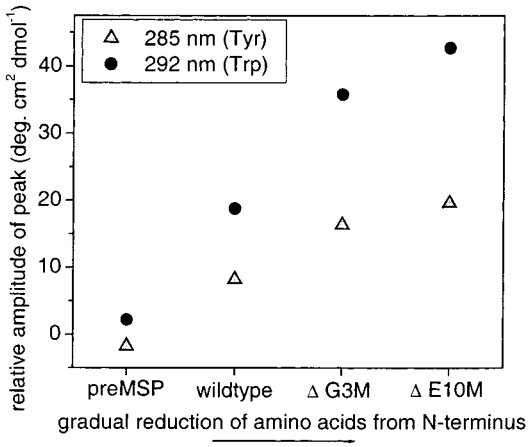

FIGURE 7: Relative amplitude of tyrosine and tryptophan peaks in the near-UV CD spectra presented in Figure 6. Each point was calculated from the corresponding spectrum as a difference between the value of mean residue ellipticity at 292 or 285 nm, respectively, and the value of mean residue ellipticity at 280 nm.

acid peaks in near-UV CD spectra as a function of the extent of modification of the N-terminal sequence of MSP is summarized quantitatively in Figure 7.

UV absorption spectroscopy can also provide information about structural changes in proteins. It is known that if the environment of an aromatic amino acid is made more hydrophobic, the amplitude of characteristic peaks of UV absorption spectra will increase. On the contrary, full exposure of aromatic amino acids to a hydrophilic environment leads to the disappearance of characteristic peaks (*51*) because they are shifted to the blue. The UV spectrum of wild-type MSP has a clearly distinguished characteristic shoulder at 293 nm, arising from tryptophan absorption (*51*). Results of UV absorption measurements (Figure 8) are consistent with the results presented above (near-UV CD spectra). Elongation of the N-terminal region (preMSP) causes the disappearance of the tryptophan shoulder in the UV absorption spectrum. On the other hand, N-terminal truncation (ΔG3M and ΔE10M) leads to an increased intensity of the 293 nm shoulder (Figure 8A). This effect became even more evident when difference spectra of preMSP minus wild type, ΔG3M, and ΔE10M, respectively, were calculated (Figure 8B).

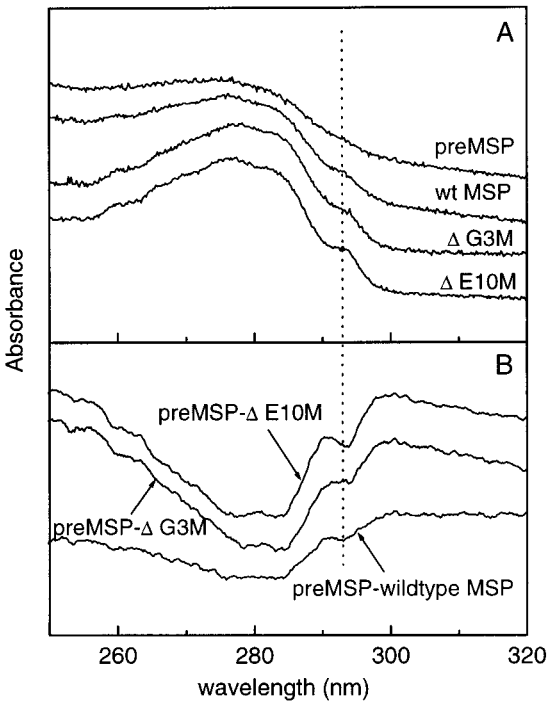

FIGURE 8: (A) UV absorption spectra of precursor, wild type, and N-terminal deletion mutants of MSP. The concentration of proteins was 10 μM in 10 mM KH_2PO_4 buffer, pH 6.0. Experimental conditions were as follows: scan width, 320−250 nm; temperature, 25 °C; path length, 1 cm; sample volume, 1 mL. (B) Difference spectra of preMSP minus wild-type MSP, ΔG3M, and ΔE10M, respectively. Each spectrum was smoothed by the adjacent averaging method (mean value of four points as the new data point). The vertical dotted line marks the position of 293 nm.

Table 4: Binding of PreMSP and ΔG3M to Intact PSII Membranes[a]

protein	addition to reconstitution mixtures (mol/mol of PSII) (%)	binding to intact PSII
preMSP	2	ND[b]
preMSP	10	144
ΔG3M	2	116
ΔG3M	10	172

[a] Because preMSP comigrates with the CP43, the integrated intensity of the Coomassie-stained 43 kDa band was evaluated in the case of preMSP; the native CP43 (for preMSP) and MSP (for ΔG3M) contents, respectively, of intact PSII membranes were used as control (100%) protein levels. The data are presented as a percentage of control values; CP47 was used as an internal standard for normalization of MSP and CP43 bands. [b] ND, not determined.

DISCUSSION

It has been established by cross-linking experiments that the N-terminal domain of MSP, extending from ^{1}E to ^{76}K, is responsible for an interaction with PSII that is localized on the large, extrinsic "E" loop of CP47 (*32*). This information is extended and refined by the demonstration that the first 16 or 18 amino acid residues at the N-terminus are essential for MSP binding (*36*) to PSII. The results presented in this paper on an extension of, or selective truncations to, the N-terminus of MSP reveal new aspects about the relationship between this domain of the protein and its functional and structural features.

Extending the MSP N-terminus to its precursor form by retention of the transit peptide sequence yields a protein with mixed functional properties. First, preMSP clearly has the ability to bind specifically to the sites on PSII from which the mature protein activates high turnover rates of the OEC and produces long-term stability of this activity. This functional rebinding occurs with lower affinity than does binding of the wild-type protein; more than 3 mol (instead of 2) of preMSP/mol of PSII was required to achieve binding of 2 mol of MSP/mol of PSII (Figure 1B). At the present time, we cannot determine the origin of this effect. Nonspecific binding of preMSP is also evident from the data shown in Figure 1B. Approximately 3 mol of preMSP was bound to PSII when 10 mol of precursor protein was incubated with 1 mol of urea-washed PSII membranes. This indicates that about one copy of preMSP was bound nonspecifically. Additional experiments confirm that preMSP is capable of nonspecific binding to PSII; MSP-containing intact PSII was reconstituted with preMSP (Table 4), and nonspecific binding of preMSP was observed in this system as well. There are two likely explanations for this result.

One is formation of preMSP dimers in solution, as revealed by size-exclusion chromatography (see Table 2). However, because saturation of activity by reconstitution with preMSP is consistent with high-affinity binding of monomeric protein to specific sites (see Figure 2), it is probable that the nonspecific binding we have detected is an artifact attributable to contamination by preMSP dimers.

The second possibility for nonspecific binding might arise from an increase in electrostatic interactions between preMSP and PSII membranes, because the transit peptide sequence adds eight positive and four negative charges not present in the wild-type protein (data not shown). It is important to note that the extension of mature MSP by the 84 amino acid transit peptide does not have an effect on functional binding of protein (Figure 2). This fact has a significant consequence for structural considerations concerning the folding of MSP when bound to PSII. It seems likely that the N-terminus of MSP is exposed, and located on the surface of PSII, rather than buried either in the tertiary structure of MSP or in a hydrophobic domain of PSII.

The first three amino acids, EGG, from the N-terminus of MSP do not appear to be critical for function, as shown by the reconstitution data obtained after mixing of usw-PSII with the truncation mutant ΔG3M (Figure 4). In comparison to wild-type protein, this MSP mutation facilitates binding to PSII membranes (Figure 3). When 10 mol of ΔG3M was incubated with 1 mol of PSII, about an additional 4 mol was bound in addition to the 2 mol of MSP normally bound to the functional site on PSII. Because this level of nonspecific binding is unprecedented, several control experiments were carried out. Contamination by unbound ΔG3M was eliminated by extensive washing of reconstituted PSII membranes; this treatment failed to lower the amount of bound protein (data not shown). An overestimation of protein binding due to background contamination by the heavily stained LHCII band was eliminated by altering the electrophoretic conditions (10% acrylamide gel, instead of 13.5%, was used to improve band resolution; data not shown), and protein aggregation was eliminated by centrifugation of MSP solutions before reconstitution and by taking optical density readings at 550 nm to detect turbidity. To further characterize nonspecific binding of ΔG3M, two experiments were carried out. The first experiment showed that increased binding to urea-washed PSII membranes occurs even in the presence of higher ionic strength (data not shown). Results of the second experiment are presented in Table 4 and show reconstitution of intact PSII membranes with ΔG3M. Intact PSII membranes possess specifically bound native MSP, and only nonspecific sites are available during reconstitution with the mutant. As results in Table 4 show, about 1.5 mol of ΔG3M was nonspecifically bound to PSII when 10 mol of MSP/mol of PSII was present in the reconstitution mixture. Size-exclusion chromatography indicates that the ΔG3M mutant exhibits a lower molecular mass than wild type, and this feature could account for its nonspecific binding by allowing the slightly smaller, more compact mutant protein access to nonfunctional binding sites in PSII. Alternatively, deletion of a negative charge (carried by ^{1}E) from MSP might change electrostatic interaction between MSP and PSII. However, removal of a negatively charged residue (^{246}E) from the MSP C-terminus (*11*) caused no change in either molecular size or MSP binding. Despite the nonspecific binding behavior of these proteins, the high O_2 evolution activities reconstituted by preMSP and ΔG3M provide evidence that both of these MSP variants retain high-affinity interactions with specific binding sites.

In contrast to preMSP and ΔG3M, consequences of removing 10 amino acid residues from the N-terminus (ΔE10M) produced a negative effect, but not total loss, of binding and reconstitution activity of this mutant protein. Only 1 mol ΔE10M/mol of PSII could be shown to rebind to PSII when between 2 and 10 mol of the mutant protein was incubated with 1 mol of usw-PSII. Deletion of ten N-terminal amino acids probably caused the observed decrease in activity and loss of binding of one copy of MSP (Figures 3 and 4). The N-terminus of MSP is a highly conserved amino acid sequence (*31*, *36*, *37*), so deletion of the domain ^{4}K−^{10}E has probably eliminated specific binding of one copy of ΔE10M. The ΔE10M mutant has a smaller molecular mass than the ΔG3M (see Table 2). If increased compactness of ΔE10M has any positive effect on protein binding, this effect has been erased by loss of important amino acid residues. It is likely that the ΔE10M mutation influences the first step of the proposed two-step mechanism of MSP function (*52*), i.e., it affects MSP binding to PSII, rather than the subsequent conformational change that accompanies folding and assembly of MSP into a functional interaction with PSII membranes. Last, binding of ΔE10M is not cold sensitive (data not shown), unlike the V235A mutant of MSP, which binds tightly to only one of two specific sites (*21*), so temperature sensitivity is not responsible for loss of binding of one MSP copy.

A structural prediction based on the primary sequence of MSP suggests that two secondary structure components lie within the first 20 N-terminal amino acid residues of MSP. Bricker and Frankel (*53*) propose that two β-sheets are located in the domain between ^{6}K and ^{9}D and between ^{12}K and ^{20}K. A more recent prediction by the PSIPRED server (see Materials and Methods) suggests the presence of two small α-helixes, one situated between ^{8}Y and ^{11}I and the second between ^{16}Y and ^{19}V. Truncation of three N-terminal residues (ΔG3M) would not affect either secondary structure component (whether α-helix or β-sheet). Deletion of first 10 amino acids in ΔE10M, however, would remove the first predicted β-sheet or delete one-half of the first predicted α-helix. If secondary structure at the N-terminus is necessary for high-affinity, functional binding of MSP, this would explain the results obtained for the 10 amino acid truncation we report here. Confirmation of this hypothesis needs, however, further investigation. Other N-terminal mutations of MSP are now being characterized.

Enami et al. (*54*) reported the formation of an intramolecular cross-link between Glu 246 and Lys 48 by EDAC when MSP is in solution, which suggests that the C- and N-termini of MSP may be in close proximity to one another. The only Trp residue of MSP is located at position 241, near the C-terminus (*14*). The data obtained from near-UV CD and UV absorption spectra (Figures 5−8) show that aromatic amino acid residues become more solvent exposed when wild-type MSP is extended at the N-terminus (preMSP), whereas the same methods indicate that these residues are shielded from solvent in a more hydrophobic environment when wild-type MSP is truncated at the N-terminus (ΔG3M, ΔE10M). For ΔE10M this effect is more apparent than for

ΔG3M. Combining these facts with the order of proteins as presented from top to bottom in Figures 5 and 6, it can be hypothesized that the gradual N-terminal truncation of amino acid residues from MSP may lead to closer contacts between the C-terminus and the N-terminus, leading to a greater overall compactness of the protein, which is in agreement with gel filtration data. Therefore, a direct consequence of N-terminal truncation of wild-type MSP would be that the aromatic residues in the compact truncation mutants are sequestered in a more hydrophobic environment. It is interesting that a completely opposite behavior in solution occurs when wild-type MSP is truncated at the C-terminus. Gradual deletion of ^{246}E, ^{245}L, and ^{244}Q from the C-terminus leads to solvent exposure of aromatic amino acid residues as shown by loss of near-UV CD peaks and to an increase in the apparent size of MSP in solution (see ref *14*). Although the spectroscopic features of Trp 241 may only monitor structural changes related to the C-terminus of MSP, the features arising from Tyr and Phe monitor the overall structure of MSP because these residues are distributed throughout the protein's sequence.

It is noteworthy that the secondary structure of N-terminally modified MSP species is preserved and is not influenced by changes in overall protein compactness (Table 3). Therefore, the secondary structure of MSP is not sensitive to the changes at the N-terminus; for example, Eaton-Rye and Murata failed to observe changes in far-UV CD spectra of protease-truncated (16 and 18 amino acids from the N-terminus) MSP (*36*). A mutant lacking 18 N-terminal amino acids is now in preparation and should simulate the protease digestion employed by Eaton-Rye and Murata in ref *36*. An extended analysis of this mutant should provide additional information on the relationship between the N-terminus of MSP and its secondary structure (*43*, *50*, *55*).

The discovery that removal of 10 amino acids from MSP at its N-terminus eliminates binding of one copy of MSP to PSII, and negatively affects activity of samples reconstituted with a single copy of the protein, provides further biochemical evidence that two PSII-bound copies of MSP are required per reaction center for optimal functioning of the OEC. In contrast, a single bound copy of MSP is resolved in electron microscopic (*22*, *23*) and crystallographic images (*24*). Image construction predicts a globular shape for bound MSP, whereas the X-ray image of the bound protein predicts that it is cylindrical in shape. In solution, MSP is a prolate ellipsoid (*56*), and binding to PSII is predicted to confer additional secondary structure (β-sheet) on the protein (*57*). These observations are consistent with the X-ray crystal structure but not with predictions of a globular shape for bound MSP. Until there is further progress in improving the resolution of the current structures of intact PSII preparations, the basis for the variable estimates for the stoichiometry and shape of MSP, discussed above, will remain unclear.

In summary, results in this paper show that the presence of the transit peptide, or truncation by the three N-terminal amino acid residues, EGG, can induce nonspecific binding of MSP to PSII without loss of specific binding of these proteins to the functional site or sites in PSII. In the case of both of these N-terminally modified proteins, functional reconstitution of PSII is obtained with restoration of oxygen evolution rates and yields that are equivalent (preMSP) or better (ΔG3M) than results with recombinant wild-type MSP. Our results with the mutant ΔE10M, however, show that the MSP domain ^{4}K−^{10}E seems to be essential for binding of one copy of MSP to PSII and also for restoration of high rates of activity. Because elimination of 16 or 18 N-terminal amino acid residues causes complete loss of binding and function of MSP (*36*), it seems possible that the domain essential for specific binding of the second copy of MSP could be located between amino acid residues ^{11}I and ^{18}E. This question is under investigation.

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BI0118761