The Carboxyl-Terminal Tripeptide of the Manganese-Stabilizing Protein Is Required for Quantitative Assembly into Photosystem II and for High Rates of Oxygen Evolution Activity†

Scott D. Betts,‡,§ Nikos Lydakis-Simantiris,‡ Jeannine R. Ross,‡ and Charles F. Yocum*,‡,||

Department of Biology and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1048

Received June 1, 1998; Revised Manuscript Received July 22, 1998

ABSTRACT: The extrinsic manganese stabilizing protein of photosystem II is required for Mn retention by the O_2-evolving complex, accelerates the rate of O_2 evolution, and protects photosytem II against photoinhibition. We report results from studies of the in vitro reconstitution of spinach photosytem II with recombinant manganese stabilizing protein with C-terminal deletions of two, three, and four amino acids. The deletions were the result of amber mutations introduced by site-directed mutagenesis. Removal of the C-terminal dipeptide (Glu-Gln) did not diminish the ability of the manganese stabilizing protein either to rebind to or to restore high rates of O_2 evolution to photosystem II preparations depleted of the native protein. Deletion of the C-terminal tripeptide (Leu-Glu-Gln) resulted in weakened but specific binding of manganese stabilizing protein to photosystem II and minimal recovery of O_2 evolution activity. Removal of the C-terminal tetrapeptide (Gln-Leu-Glu-Gln) eliminated the ability of the subunit to interact stably with all of its available binding sites on photosystem II, as evidenced by the fact that this mutant was totally inactive in restoring O_2 evolution activity. Evidence is presented to indicate that these mutational effects on the binding and function of the manganese stabilizing protein may be due to major changes in tertiary structure. The truncation mutations lacking either the C-terminal tri- or tetrapeptide exhibit apparent size increases of 25 and 40%, respectively, when compared either to a mutant lacking the C-terminal dipeptide or to the wild-type protein.

Photosynthetic O_2 evolution by plants, algae, and cyanobacteria is dependent on the presence of Mn, Ca^{2+}, and Cl^- (*1*, *2*). Ligation of these cofactors by intrinsic protein components of the photosystem II (PSII)[1] reaction center forms the active site for H_2O oxidation, called the O_2-evolving complex, or OEC. Maximum activity and stability of this site is conferred by three extrinsic proteins (*3*, *4*). The smaller (23 and 17 kDa) of these subunits facilitate retention of Ca^{2+} and Cl^- by PSII, but appear to have no direct interaction with either inorganic cofactor. The largest subunit is a 26.5 kDa polypeptide called manganese stabilizing protein, or MSP (*3*). This protein stabilizes binding of two of four Mn atoms to the active site of H_2O oxidation, accelerates the rate of O_2 evolution, and regulates the stability of oxidation state intermediates in the water oxidizing reaction (*5*−*7*).

The essential role of MSP in stabilizing Mn retention by the OEC suggested that characterization of the binding interactions between this protein and the intrinsic subunits of the PSII reaction center might provide useful information with respect to both MSP structure and the location of the Mn cluster itself. Eaton-Rye and Murata (*8*) used limited proteolysis to show that removal of the N-terminal 16−18 amino acids from MSP caused no major changes in the protein's secondary structure as monitored by circular dichroism, yet the truncated protein was unable to rebind to PSII. Additional information on the MSP−PSII interaction came from investigations showing that the binding interaction of MSP with PSII involves the large, lumenally exposed "E" loop of the intrinsic Chl *a* binding protein called CP47. In these studies, Bricker and co-workers identified domains on CP47 which interact with bound MSP (*9*). Use of the zero-length cross-linker EDC has resulted in one or more covalent linkages between MSP and CP47 (*9*). Amino acid sequencing of the region of the cross-link by Odom and Bricker (*10*) identified the N-terminal 70 amino acids of MSP and the "E" loop of CP47 as the sites that are involved in the cross-linking reaction.

Interactions of MSP with other intrinsic subunits of PSII have proven to be more difficult to identify at the same level of resolution as the interaction between MSP and CP47 just

† This research was supported by Grants MCB-9314743 and MCB-9727777 to C.F.Y. from the Molecular Biochemistry Program of the National Science Foundation.

* Address correspondence to this author at the Department of Biology, The University of Michigan.

‡ Department of Biology.

§ Current address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

|| Department of Chemistry.

[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; CD, circular dichroism; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EDTA, (ethylenedinitrilo)tetraacetic acid, disodium salt; IPTG, isopropyl-β,D-thiogalactose; 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 expression with T7 RNA polymerase; preMSP, *psb*O gene product and precursor polypeptide of MSP; *psb*O, cDNA clone encoding preMSP; PS, photosystem; R_S, Stokes radius; SDS, sodium dodecyl sulfate; SW, PSII membranes depleted of 23- and 17-kDa polypeptides by washing with 2 M NaCl; TMA^+, tetramethylammonium; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(98)01305-1 CCC: $15.00
Published on Web 09/18/1998

described. For example, Seidler (*11*) has explored an alternative cross-linking method with MSP in which the protein in solution is first activated for cross-linking by reactions with EDC and sulfo(*N*-hydroxy)succinimide and then added to PSII to initiate formation of cross-linked products. This approach resulted in formation of cross-links to CP47 and also to another, small extrinsic subunit of PSII that has not so far been identified. Enami et al. (*12*) have used trypsin hydrolysis and peptide sequencing to identify domains of other PSII intrinsic proteins that may interact with MSP. A site in the "E" loop of the other tightly bound, Chl *a* binding antenna protein, CP43, is unmasked and exposed to trypsin digestion when MSP is extracted from PSII, and the C-terminus of the α-subunit of cytochrome *b*559 is also exposed to trypsin digestion by MSP extraction.

The data on MSP−PSII interactions that have accumulated so far can be interpreted to suggest that there is more than one site for MSP binding in PSII. This interpretation is consistent with the finding that there are two copies of MSP per PSII reaction center (*13*−*15*). It is thus plausible to assume that one copy of MSP has a strong interaction with the large extrinsic loop of CP47, while the second copy may bind to another PSII subunit [CP43, the cytochrome *b*559 α subunit, or the small intrinsic subunit that cross-links to MSP in the experiments reported by Seidler (*11*)]. Binding of two copies of MSP to different core subunits in PSII would imply that two domains in the protein are required for binding to PSII. In this context, evidence exists to indicate that the C-terminal domain of MSP is also important in binding of the protein to PSII. Chemical modification of MSP (biotinylation) has demonstrated that amino groups in the C-terminal half of MSP are buried in the PSII-bound protein (*16*). Replacement of a conserved Val residue by Ala at position 235 (13 residues from the C-terminus) affected assembly of the mutant protein to PSII at 4 °C, but not at room temperature (*17*). Drastic truncations that remove 30 or more amino acids from the MSP C-terminus [Betts, S. D. (1996) Ph.D. Thesis, University of Michigan; Seidler et al. (*18*)] eliminate the protein's ability to bind to PSII. Last, introduction of a His-tag at the C-terminus interferes with normal binding of MSP as evidenced by a requirement for additional protein to produce fully saturated binding of MSP to PSII, and by the distinctive sigmoidal appearance of the His-tagged protein's PSII binding curve (*19*).

We have used the technique of MSP overexpression in *E. coli* coupled with site-directed mutagenesis to examine the role of the protein's C-terminus in binding to PSII and restoration of activity. As we report here, the results of our experiments show that the two terminal amino acids of spinach MSP are not essential for the protein's biological activity. However, site-directed mutagenesis to remove the conserved Leu residue at position 245 dramatically reduces the ability of MSP to restore H_2O oxidation activity, and removal of Q244 abolishes MSP rebinding to PSII and as a consequence eliminates restoration of O_2 evolution activity. These phenomena can, in part, be ascribed to structural changes in mutant proteins lacking the terminal tri- and tetrapeptides. That these species have undergone a substantial structural change is evidenced by a decrease in their respective elution volumes on a calibrated size-exclusion chromatography column.

MATERIALS AND METHODS

psbO Clones and Site-Directed Mutagenesis. The plasmid constructs used here for mutagenesis and expression of spinach and *Arabidopsis psb*O were described previously (*20*, *21*). The deduced amino acid sequence of *Arabidopsis* preMSP was reported in (*20*), and that for spinach preMSP corresponded to the sequence of mature wild-type spinach MSP reported in (*22*). The phagemid pBluescript SK+ was used to produce single-stranded template DNA for oligonucleotide-directed mutagenesis (*23*) and dideoxy sequencing (*24*). The first truncation mutant was generated with *Arabidopsis psb*O before spinach *psb*O was cloned in our laboratory. The codon specifying Q245 in *Arabidopsis* MSP (Q244 in spinach MSP) was changed from CAA to a stop codon, TAA. This single nucleotide substitution also introduced a *Hpa*I restriction endonuclease site (GTTAAC). The codon for E246 in spinach MSP was changed from GAG to TAA. Similar strategies were employed to introduce stop codons that yielded the other C-terminal amino acid truncations reported in this paper. All mutations were confirmed by DNA sequencing.

Expression System and Growth Conditions. The construction of pET expression vectors with inserts of either *Arabidopsis psb*O or spinach *psb*O was described previously (*20*, *21*). These clones encode the full-length precursor protein of MSP, denoted preMSP. Overexpression of *psb*O was induced by addition of IPTG to log-phase cultures of *E. coli* strain BL21(DE3)pLysS transformed with various pET(*psb*O) plasmid constructs. All cells were cultured in LB medium supplemented with ampicillin (50 μg/mL) and chloramphenicol (25 μg/mL).

Expression and Purification of Deletion Mutants. Growth conditions were employed that promote processing of the preMSP transit peptide [rotary shaking of bacterial cultures at 150 rpm after induction of protein expression with 20−30 μM IPTG (20, 21)]. Mature, overexpressed MSP's were quantitatively solubilized from inclusion bodies by incubation in 3 M urea, and then purified by anion-exchange chromatography using a Pharmacia FPLC system fitted with Resource-Q or Mono-Q columns, also with 3 M urea in the equilibrating and eluting buffers (*21*). The C-terminal truncation mutants lacking three and four amino acids presented difficulties in purification. The urea-solubilized proteins were substantially, but not completely, purified by anion-exchange chromatography in 3 M urea as described above. Insufficient yields were achieved following removal of urea by dialysis or by dilution and subsequent anion-exchange chromatography in the absence of urea. Even though these mutant proteins are soluble in urea-free solvent, they appear to aggregate on the anion-exchange column in the absence of urea, and therefore this final nondenaturing chromatographic step was, of necessity, omitted in the purification of these mutants.

Assay and Reconstitution Systems. Photosystem II-enriched thylakoid membrane fragments and derivative preparations lacking the 17- and 23-kDa extrinsic proteins ("salt-washed") or all three extrinsic proteins ("urea-washed") were prepared from spinach leaves as described previously (*20*). Oxygen evolution activity was measured polarographically at 25 °C using a Clark-type O_2 electrode. The assay medium and conditions were described in ref *20*; the electron

acceptor was 600 μM DCBQ. Only the 4-min O_2 yield values (micromoles of O_2 per milligram of Chl) are reported here. Urea-washed PSII membranes were incubated with varying amounts of recombinant MSP according to the procedure in Betts et al. (*17*) under the conditions specified in the figure legends. Reconstitution mixtures were diluted 20-fold into the assay cuvette immediately before O_2 evolution assays. Samples were prepared for SDS−PAGE analysis by centrifugation (10 min, 12000*g*) and washing (2 volumes of assay buffer and recentrifugation) to remove unbound protein. The washed pellets were resuspended to a final concentration of 1 mg of Chl/mL.

Binding of the *Arabidopsis* mutant protein, [Q245@]MSP, was also attempted by dialysis to slowly remove denaturant. Standard reconstitution conditions were used, except the concentration of Chl was 0.1 mg/mL and urea was present (1.7 M) at the start of dialysis. Samples (800 μL) were dialyzed against 100 volumes of 0.4 sucrose, 50 mM MES (pH 6), 20 mM $CaCl_2$, 60 mM NaCl, and 5% betaine (w/v). Dialysis was performed in two 3-h steps with stirring at 22 °C in the dark.

Reconstituted, washed PSII samples were analyzed by SDS−PAGE (10% acrylamide) according to Piccioni et al. (*25*). Urea (4.7 M) was included in all gels. Under these conditions of electrophoresis, soluble, purified MSP samples are observed to migrate at slightly higher rates than MSP that is associated with PSII membranes (compare, for example, lanes "WT" and "SW" of Figure 4), presumably because of protein−protein interactions in the more complex PSII membrane samples. Coomassie-stained gels were scanned using an LKB 2222-010 UltroScan XL laser densitometer, and relative amounts of PSII-bound MSP were determined by integration of peak areas as described previously (*17*).

Size-exclusion chromatography experiments to estimate the molecular masses of wild-type and mutant proteins were carried out using a Superose 12 column attached to a FPLC system. The column was equilibrated with 20 mM Bis-Tris (pH 6.4) containing 100 mM NaCl. The column was calibrated with a set of standard proteins [aprotinin (6.6 kDa), cytochrome *c* (13 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa)] obtained from Sigma Chemical Co. The void volume of the column was determined from the volume required to elute Blue Dextran. Each molecular mass determination was repeated twice, and the average elution volume was used to estimate the molecular mass of a particular protein.

RESULTS

Table 1 presents the sequences of the mutations described in this paper. By combining data from recombinant spinach and *Arabidopsis* MSP species, it has been possible to systematically examine the effect on biological activity of removing one amino acid at a time from the C-terminus of MSP. The results of experiments presented elsewhere show that recombinant spinach and *Arabidopsis* MSP's exhibit identical activities with respect to both binding and activity reconstitution assays when a MSP-depleted PSII preparation isolated from spinach was employed (*26*). Since *Arabidopsis* MSP lacks the C-terminal Gln residue (Table 1) present in the spinach protein, the published reconstitution results with the former species (*26*) demonstrate that the C-terminal Gln residue of spinach MSP is not required for the protein's activity.

Table 1: Aligned C-Terminal Amino Acid Sequences of Wild-Type and Mutant Recombinant MSP[a]

protein	sequence
wild type (spinach)	W Y A Q L E Q@
wild type (*Arabidopsis*)	− − G − − − @
E246@ (spinach)	− − − − − − @
L245@ (spinach)	− − − − − @
Q245@ (*Arabidopsis*)	− − G @
Q244@ (spinach)	− − − @

[a] Dashes indicate conserved amino acid residues in all sequences; @ symbolizes a naturally occurring or engineered stop codon in the MSP sequence. The apparent discrepancy in numbering of an otherwise highly conserved amino acid sequence (for example, L245 in Spinach; Q245 in *Arabidopsis*) is due to deletion of a single amino acid (P4) in spinach MSP.

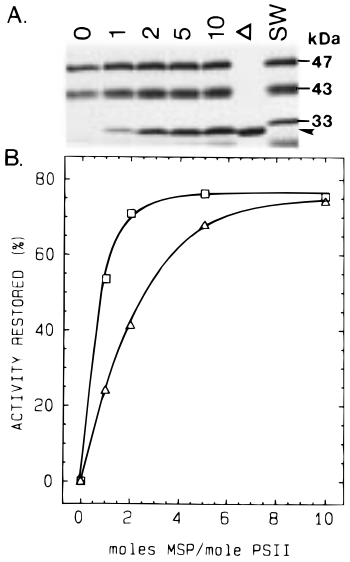

FIGURE 1: Reconstitution of PSII with [E246@]MSP. (A) Urea-washed PSII samples were reconstituted with the indicated molar ratios of [E246@]MSP to PSII. After incubation at 22 °C, unbound protein was removed by centrifugation and washing. Triangle, [E246@]MSP; SW, control salt-washed PSII with native MSP. The arrowhead on the right indicates that the truncated protein has an increased electrophoretic mobility. (B) Unwashed reconstitution mixtures were assayed (4 min O_2 yield) after 30 min reconstitution incubation at 4 °C (triangles) and again after an additional 1 h at 22 °C (squares). The control (100% yields) with wild-type MSP was 10.8 μmol of O_2/mg of Chl.

As a next step in analyzing the MSP C-terminus, the codon for E246 in spinach *psb*O was changed to a stop codon to eliminate the C-terminal dipeptide, E-Q, from the protein. The resulting recombinant protein, E246@, was overexpressed and purified as described under Materials and Methods. Results from representative experiments to assess the ability of E246@ to rebind to PSII and to reconstitute activity are presented in Figure 1. Increasing amounts of E246@ MSP were incubated with PSII for 30 min on ice followed by an additional 60 min at room temperature.

Figure 1A presents a gel showing the rebinding of the protein to PSII. Unbound MSP was removed by centrifugation and washing of PSII membrane pellets. As can be seen, in comparison to the wild-type protein (lane SW of Figure 1A), the mutant protein exhibits a slightly faster migration rate through the SDS−polyacrylamide gel than does the corresponding wild-type protein. This behavior is consistent

with a reduction in size and/or charge of the mutant protein. Figure 1A also shows that rebinding of MSP to PSII appears to be largely unaffected by the deletion mutation. Addition of 2 mol of protein/mol of PSII reaction center, followed by washing and electrophoresis, shows that the amount of protein retained by PSII is similar to that detected in the native PSII preparation.

Figure 1B shows the oxygen evolution activity of reconstitution mixtures after 30 min on ice (triangles) and again after an additional 1 h at room temperature (squares). These assays were taken before centrifugation to remove unbound protein. Here, it can be seen that full reconstitution of activity at room temperature occurs, as expected, at 2 mol of MSP/mol of PSII reaction center (*13*−*15*), whereas a similar restoration of activity at the lower temperature requires incubation with approximately a 2−3-fold excess of E246@ MSP over what is required at 22 °C. These data show that residue E246 is not required for the biological activity of MSP. Deletion of E246 does not block restoration of activity to wild-type levels, but this amino acid does influence the ability of the protein to bind productively to PSII at 4 °C. A similar behavior has been previously reported for the mutation V235A (*17*).

To further assess the function of E246, we created another mutation that created a charge reversal (E246K) at this position in the MSP sequence. The protein bearing this mutation rebinds normally to PSII, and activity restoration at room temperature is similar to that observed with wild-type MSP and with E246@ (see Figure 1). Taken together, the results just described indicate that E246 is dispensable with respect to MSP binding to PSII at room temperature, and for reactivation of the OEC as well.

Proceeding systematically to the next residue in the C-terminal sequence of spinach MSP, we examined the consequences of deleting the tripeptide L-E-Q. The results of rebinding and activity reconstitution assays using the truncation mutant L245@ are presented in Figure 2. Data produced in the binding assay (Figure 2A) show that the L245@ mutation has a substantial effect on MSP binding to PSII (circles in Figure 2B). An apparent saturation of binding at the wild-type level can be achieved only upon incubation of between 17 and 21 mol of L245@ per mole of MSP-depleted PSII. The activity assays (squares) produce results that indicate that even when amounts of L245@ stoichiometric with the amounts of native wild-type protein are rebound to PSII, only very small amounts of activity (<20%) are recovered. Therefore, these data indicate that deletion of the C-terminal tripeptide produces two rather dramatic effects on MSP function. First, the mutant protein can rebind to PSII, but excessive stoichiometries of added protein are required to achieve binding at wild-type levels, indicating a defect in the ability of the protein either to assemble into PSII or, once assembled, to remain bound. Second, the data of Figure 2 show that when a native stoichiometry of bound MSP (2 mol/mol of reaction center) is achieved, no more than about 20% of wild-type activity is recovered.

A further truncation of spinach MSP was carried out by producing and overexpressing the mutant Q244@. Results of experiments to analyze the consequences of this mutation are shown in Figure 3. The data show that the additional truncation produces an even greater effect on MSP binding

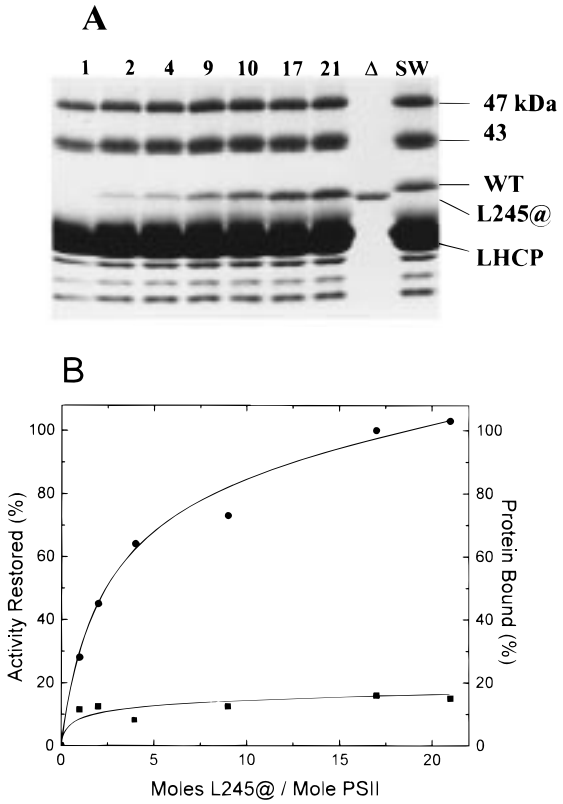

FIGURE 2: Reconstitution of PSII with [L245@] spinach MSP at 22 °C. (A) SDS gel showing rebinding of mutant protein to uswPSII membranes. Numbers above lanes represent moles MSP per mole of PSII in the incubation step before washing to remove unbound protein. (B) Circles represent the amount of MSP bound relative to a wild-type control sample prepared by incubating MSP-depleted PSII with 5 mol of wild-type MSP/mol of PSII reaction center, followed by washing to remove excess, nonspecifically bound protein. The amount of wild-type MSP retained by PSII under these conditions is about 2 mol/mol of reaction center (*17*). The squares represent the activity yield reconstituted by L245@ as a percent of the activity of a sample reconstituted with saturating wild-type MSP (12.8 μmol of O_2/mg of Chl).

than does the truncation mutation L245@. At the highest concentrations of protein used, no more than about 50% of wild-type levels of MSP rebinding can be obtained (Figure 3B, circles). Also shown in Figure 3B are the results of activity assays conducted on PSII samples reconstituted with spinach Q244@ (squares). Insofar as we can determine from a series of experiments with the mutant protein, there is no detectable restoration of O_2 evolution activity, even when there is clear evidence for rebinding of about 50% of the amount of wild-type levels of MSP to PSII. In the absence of activity restoration, we cannot be certain that the binding we observe with mutant does not arise from adventitious, rather than specific, reassociation of MSP with PSII. The gel lane of Figure 3A marked by △ reveals the level of contaminants in this preparation that were impossible to remove by our purification methods. We estimate that these polypeptides constitute a contamination of about 10% (data not shown).

The effect of the equivalent deletion from the C-terminus of *Arabidopsis* MSP, producing the mutant Q245@ (Table 1), was also tested. Removal of the two amino acid sequence L-E from the C-terminus of *Arabidopsis* MSP completely abolished the ability of the overexpressed protein to bind to spinach PSII (Figure 4). The wild-type *Arabidopsis* protein

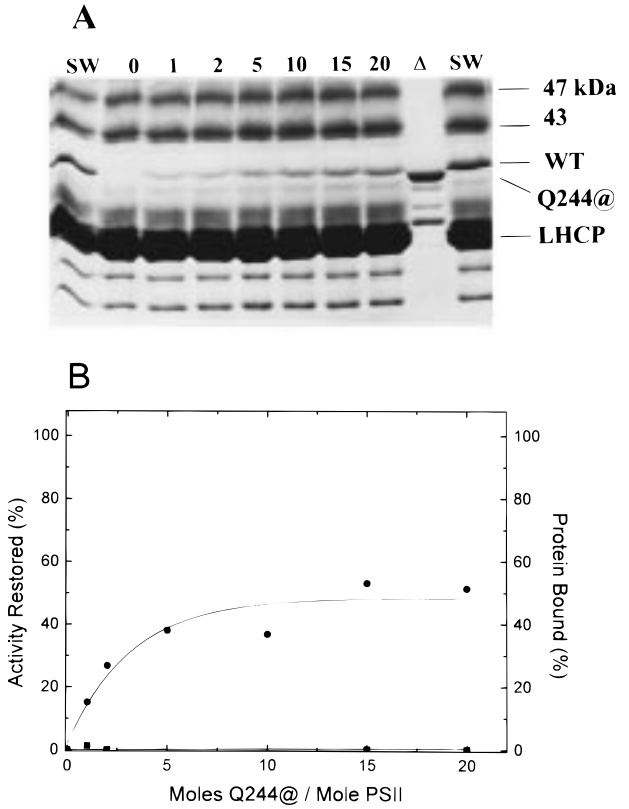

FIGURE 3: Reconstitution of PSII with [Q244@] spinach MSP at 22 °C. Symbols, conditions, and activities are as given in the legend to Figure 2.

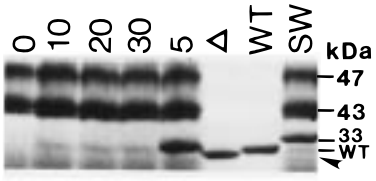

FIGURE 4: SDS−PAGE analysis of urea-washed PSII following dialysis in the presence of [Q245@] *Arabidopsis* MSP. The molar ratio of [Q245@]MSP to PSII is indicated above the lanes (10, 20, and 30). As a control, an identical PSII sample was dialyzed with 5 mol of wild-type *Arabidopsis* MSP per mole of PSII. Unbound protein was removed by centrifugation and washing. Other symbols are as follows: △, [Q245@]MSP used for reconstitutions; WT, wild-type *Arabidopsis* MSP used for the control reconstitution; SW, salt-washed spinach PSII preparation. The arrowhead in the figure indicates the increased electrophoretic mobility of the mutant protein.

(Figure 4, lane marked WT) migrated with increased electrophoretic mobility as compared with wild-type spinach MSP shown in the right lane of the gel in Figure 4. The *Arabidopsis* truncation mutation (labeled Δ in the figure) migrates at a slightly higher rate than does native *Arabidopsis* MSP. We also attempted assembly of *Arabidopsis* [Q245@]-MSP under other conditions without success. These included renaturation of the mutant protein by dialysis before mixing with urea-washed PSII and also by combined refolding/assembly by dilution into a urea-washed PSII suspension. Failure of the *Arabidopsis* MSP mutant Q245@ to bind to spinach PSII provides additional evidence for an essential role of the C-terminal sequence of the spinach protein in conferring specificity for photosystem II.

We observed a faint band of protein comigrating with MSP which was visible in each washed sample of PSII that had been mixed with a large excess of [Q245@]MSP. This band did not obviously increase in staining intensity with increasing amounts of added protein, and we suggest that it most likely represents residual [Q245@]MSP associated nonspecifically with PSII. Regardless of its identity or origin, it clearly bound to PSII only in trace amounts and did not activate oxygen evolution.

Table 2: Molecular Masses and Stokes Radii of Wild-Type and Mutant Spinach MSP's Estimated from Size-Exclusion Chromatography Elution Volumes

protein	molecular mass (kDa)	Stokes radius (Å)[a]
wild-type MSP	41	31.0
E246@	42	31.1
L245@	52	33.5
Q244@	58	34.5

[a] Standards used were bovine serum albumin (R_S = 35.5 Å), carbonic anhydrase (R_S = 20.1 Å), and cytochrome *c* (R_S = 16.3 Å) (*28*).

To further explore the consequences of the truncation mutations described here, we carried out experiments in which these proteins were characterized by size-exclusion chromatography, as described under Materials and Methods. Results of these experiments show that the two spinach truncation mutations (L245@, Q244@) produce proteins that elute from the size-exclusion column with diminished volumes as compared to the wild-type protein (data not shown). In contrast, E246@ does not produce a significant difference in the elution volume compared to wild-type MSP. To ensure that the size increase for these mutations is not due to fortuitous aggregation of the protein on the column, additional experiments were done in which urea was included in the elution buffer at a concentration of 3 M. This concentration, which is sufficient to dissolve MSP inclusion body aggregates, yielded a slight increase in the estimated molecular mass (data not shown), rather than a decreased size, as would be expected if aggregation of the protein were occurring on the column.

The actual estimates of molecular masses and of Stokes radii derived from gel filtration experiments are presented in Table 2. These data indicate that C-terminal tri- and tetrapeptide truncations of MSP lead to changes in solution structure that produce proteins that are less compact than the wild-type species. The data presented in Table 2 suggest as well that wild-type MSP is itself larger than would be predicted on the basis of its calculated molecular mass (26.5 kDa). The estimated Stokes radii shown in Table 2 are typical of globular proteins with molecular masses in the range 40−45 kDa (wild type and E246@), and 55−65 kDa (L245@ and Q244@)(*28*, *29*). Previous experiments with MSP have shown that the protein exhibits an anomalously large size by electrophoresis on polyacrylamide gels under denaturing conditions (*30*) and by gel filtration under native conditions (*21*, *31*).

DISCUSSION

In addition to an essential role for the first 16 amino acids of MSP in binding to PSII (*8*), the only other structural feature in the protein that previously has been shown to be required is the intramolecular disulfide bridge between residues C28 and C51 (*27*) (higher plant numbering), the only two Cys residues in all MSP's examined so far. Reduction by mercaptoethanol of this disulfide in isolated spinach MSP denatured the protein and eliminated its ability

to rebind to PSII (*30*). Subsequent reoxidation of reduced MSP regenerated the disulfide bridge and restored the protein's binding ability. We have shown that deletion of the disulfide bridge by eliminating the Cys residues using site-directed mutagenesis does not interfere with PSII binding or MSP function (*21*). We have interpreted this result to indicate that the −S−S− bridge is necessary to stabilize MSP structure in solution. We have also completed a characterization of the effects on MSP activity of retention of the signal peptide and of a mutation placing a Met residue ahead of the Glu at the N-terminus of the protein (Betts et al., manuscript in preparation). Although these mutations modify binding efficiency, they do not abolish it.

Owing to its inaccessibility to biochemical probing, less is known about the C-terminus of MSP than is known about the N-terminus of the protein. A domain in the C-terminal half of MSP, Lys159−Lys186, was protected from biotinylation when bound to PSII, and another long sequence of amino acids, Lys190−Lys236, extending almost to the C-terminus, was not protected from biotinylation by association with PSII (*32*). Extension of the C-terminus of spinach MSP with six His residues weakened, but did not abolish, binding of the His-tagged protein to PSII (*19*). Other than these observations, nothing was known previous to this report about the involvement, if any, of the C-terminal amino acid sequence of MSP in binding to PSII, and of its possible function in promoting recovery of H_2O oxidation.

Results from reconstitution studies reported here, using truncation mutants of MSP, indicate that two amino acids at the C-terminus proper may be deleted from the spinach protein without major effects on the ability of MSP to rebind to PSII and reconstitute O_2-evolution activity. The results of these experiments indicate that neither Q247 nor E246 is absolutely essential for the MSP solution structure that is capable of rebinding to the proper sites on PSII, nor for the protein's ability, once bound, to restore efficient electron-transfer function to the OEC. The data of Figure 1 do show that removal of E246 weakens MSP binding at low temperature, but this impairment can be compensated for by addition of an excess of the mutant protein, a finding similar to that obtained with the temperature-sensitive mutant V235A (*17*). These mutations appear to affect MSP assembly; once assembled, the subunit remains bound under normal storage and assay conditions. Therefore, we would conclude that while E246 may be involved in some way in the productive folding and assembly of MSP, this residue is not sufficiently important so as to create a major disruption of MSP function upon its deletion.

Substantial alterations in MSP activity are observed on deletion of the tri- (L-E-Q) and tetrapeptides (Q-L-E-Q) from spinach MSP, and the corresponding tetrapeptide in *Arabidopsis* (the Q245@ mutation that removes Q-L-E). These deletions either greatly weaken (L245@, Q244@ in spinach) or abolish (Q245@ in *Arabidopsis*) MSP binding. As can be seen from the data in Figure 2, the mutant protein L245@ can be shown to exhibit biphasic binding behavior when added to PSII, and these experiments ultimately require the presence of a large excess (ca. 15−20 mol/mol of PSII reaction center) of the mutant protein during the incubation step with PSII. After washing to remove nonspecifically bound protein, the ratio of bound L245@ MSP to PSII reaction centers is in fact equivalent to that observed for wild-type MSP. However, when polarographic assays of such samples are carried out, the results show that only minimal levels (<20%) of activity are recovered.

The consequences of removing Q244 (spinach) or Q245 (*Arabidopsis*) are even more severe. These mutations drastically reduced or eliminated the ability of MSP to interact productively with its binding sites on spinach PSII. These deletions are also distinguished from other MSP mutations that we have described here and elsewhere in that they appear to partially or fully impair the ability of MSP to recognize all of its binding sites on PSII.

The models for MSP secondary structure content proposed by Xu et al. (*33*) and by Shutova et al. (*34*) hypothesize that MSP contains substantial (33−37%) amounts of β-sheet, based on the far-UV CD spectrum of the protein. The C-terminus is predicted to be one of the regions which is comprised of β-sheet, so it is possible that the defects created by deletion of Leu and Gln from the C-terminus are related to disruption of a secondary structural domain that is essential either for MSP binding to PSII or for formation of the MSP tertiary structure in solution that can bind effectively to PSII. The data from size-exclusion chromatography experiments (Table 2) provide some insights into the nature of the defects created by the C-terminal truncations of L245 and Q244. The increased sizes of these mutant proteins would suggest that some change in protein folding or stability is caused by the C-terminal truncations, so it is possible that the C-terminal tri- and tetrapeptides of MSP are required for the solution structure of MSP that assembles efficiently into PSII. If this is so, then these amino acid residues may not play a role in the direct formation of either H-bonds or hydrophobic interactions with amino acid residues associated with the intrinsic subunits of the PSII reaction center. Rather, these amino acids may be essential for formation of the MSP solution structure whose conformation is required for correct docking with the binding sites on intrinsic protein components of PSII, and for subsequent changes in MSP conformation that may be necessary for productive assembly of the protein into PSII. This suggestion is in accord with the intramolecular cross-linking data of Enami et al. (*35*), who found that E246 of MSP forms an EDC-catalyzed cross-link to K48 in MSP in solution, whereas in the PSII-bound protein, E246 cross-links to K190. The authors propose that the shift in cross-linking could indicate that MSP changes conformation on binding to PSII, a phenomenon that has been detected by the FTIR isotope editing experiments of Hutchison et al. (*36*).

It is significant that the estimated sizes of two of the C-terminal truncation mutants (L245@ and Q244@ of Table 2) are very similar to the size estimated for native MSP dissolved in 2.5−3 M urea (*21*). This urea concentration is optimal for forcing the release of MSP from its PSII binding sites. It is therefore somewhat surprising that these truncated proteins, despite their increased sizes, retain some capacity to rebind to PSII. On account of the disparity in these observations, we have to consider the possibility that the truncations have induced unfolding of a structural feature or domain present in the unassembled wild-type protein that normally resists unfolding in 2.5−3 M urea. In fact, we are unable to find a precedent for retention of binding function in any protein subunit whose overall size has changed as much as we estimate to be the case for L245@ (+26%) and

Q244@ (+40%). This alteration in size, whatever its nature, is apparently sufficient to produce a substantial impairment in the assembly or stable binding of both copies of a truncated protein to its respective pair of binding sites in PSII. This is consistent with the results (*35*, *36*) that indicate a conformational change occurs in MSP during binding to PSII.

The hydrodynamic properties of the MSP mutants we describe here, when taken together with our earlier results (*21*) on analytical gel filtration behaviors of MSP and of mutant proteins, cannot be reconciled with the behavior that would be expected of a globular protein whose calculated molecular mass (from the DNA sequence) is on the order of 26.5 kDa. The present data extend the original characterization of MSP's anomalous behavior, first reported in experiments using SDS−polyacrylamide gel electrophoresis (*31*). Proteins exhibiting abnormally high molecular masses during electrophoresis, or on gel filtration columns, have been previously documented, and a survey of the properties of these proteins, in light of the emerging properties of MSP, is instructive. Weinreb et al. (*29*) list the properties of a number of proteins, including microtubule-associated proteins (MAPs), proteins that bind to calmodulins, kinase, and phosphatase inhibitors, and other proteins all of whom exhibit lower mobilities on SDS−polyacrylamide gels, and elution volumes on gel filtration chromatography that are inconsistent with their calculated molecular masses. The majority of these proteins have acidic p*I*'s, the biological function of most of them is to participate in protein−protein interactions, and, where available, data from circular dichroism spectroscopy indicate the presence of substantial amounts of random coil. In addition, at least in the case of certain of these proteins (*29*), an extended rather than globular solution structure is observed. This may explain the behavior of MSP and the truncation mutants in size-exclusion chromatography experiments, and the anomalous values obtained for the Stokes radii of the mutant and wild-type proteins (Table 2). Manganese stabilizing protein [pI = 5.2 (*31*)] has been proposed to contain large amounts of random coil, based on data from circular dichroism experiments that were used by two groups to deconvolute these results into predictions of secondary structure content (*33*, *34*). Results of cross-linking experiments show clearly the existence of a close interaction between MSP and other PSII proteins. On the basis of these observations, at least, it would appear that MSP belongs to the same family as the other proteins described above. For these proteins, it is suggested that the unusual gel filtration behavior that produces anomalously large molecular mass estimations arises from the very unusual solution structures of these proteins, which have been proposed to be either "natively denatured" (*37*) or, more recently, "natively unfolded" (*29*). Such behavior on the part of MSP would be consistent with the recent demonstration that upon binding to PSII, the protein undergoes a structural change involving a net gain of secondary structure (*36*). Experiments are in progress to further characterize the solution behavior of MSP to determine in more detail whether it is closely allied with other proteins that are natively unfolded in solution.

REFERENCES

1. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
2. Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1−15.
3. Seidler, A. (1996) *Biochim. Biophys. Acta 1277*, 35−60.
4. Ghanotakis, D. F., and Yocum, C. F. (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 41*, 255−276.
5. Miyao, M., and Murata, N. (1983) *FEBS Lett. 164*, 375−378.
6. Miyao, M., and Murata, N. (1984) *FEBS. Lett. 170*, 350−354.
7. Miyao, M., Murata, N., Lavorel, J., Maison-Peteri, B., Boussac, A., and Etienne, A.-L. (1987) *Biochim. Biophys. Acta 890*, 151−159.
8. Eaton-Rye, J. J., and Murata, N. (1989) *Biochim. Biophys. Acta 977*, 219−226.
9. Bricker, T. M., Odom, W. R., and Quierolo, C. (1988) *FEBS Lett. 231*, 111−117.
10. Odom, W. R., and Bricker, T. M. (1992) *Biochemistry 31*, 5616−5620.
11. Seidler, A. (1996) *Eur. J. Biochem. 242*, 485−490.
12. Enami, I., Tohri, A., Kamo, M., Ohta, H., and Shen, J.-R. (1997) *Biochim. Biophys. Acta 1320*, 17−26.
13. Leuschner, C., and Bricker, T. M. (1996) *Biochemistry 35*, 4551−4557.
14. Xu, Q., and Bricker, T. M. (1992) *J. Biol. Chem. 267*, 25816−25821.
15. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1997) *Biochemistry 36*, 4047−4053.
16. Frankel, L. K., and Bricker, T. M. (1992) *Biochemistry 31*, 11059−11064.
17. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1996) *Biochemistry 35*, 6302−6307.
18. Seidler, A., Roll, K., and Michel, H. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 409−412, Kluwer Academic Publishers, Dordrecht.
19. Seidler, A. (1994) *Protein Eng. 7*, 1277−1280.
20. Betts, S. D., Hachigian, T. M., Pichersky, E., and Yocum, C. F. (1994) *Plant Mol. Biol. 26*, 117−130.
21. Betts, S. D., Ross, J. R., Hall, K. U., Pichersky, E., and Yocum, C. F. (1996) *Biochim. Biophys. Acta 1274*, 135−142.
22. Tyagi, A., Hermans, J., Steppuhn, J., Jansson, Ch., Vater, F., and Herrmann, R. G. (1987) *Mol. Gen. Genet. 207*, 288−293.
23. Kunkel, T. A., Bebenek, K., and McClary, J. (1991) *Methods Enzymol. 204*, 125−139.
24. Sanger, R., Nicklen, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
25. Piccioni, R., Bellemare, G., and Chua, N.-H. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B., and Chua, N.-H., Eds.) pp 985−1014, Elsevier Biomedical Press, Amsterdam.
26. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1995) in *Photosynthesis: From Light to Biosphere* (Mathis, P., Ed.) Vol. II, pp 381−384, Kluwer Academic Publishers, Dordrecht.
27. Oh-oka, H., Tanaka, S., Wada, K., Kuwabara, T., and Murata, N. (1986) *FEBS Lett. 197*, 63−66.
28. Sorenson, B. R., and Shea, M. (1996) *Biophys. J. 71*, 3407−3420.
29. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996) *Biochemistry 35*, 13709−13715.
30. Tanaka, S., and Wada, K. (1988) *Photosynth. Res. 17*, 255−266.
31. Kuwabara, T., and Murata, N. (1979) *Biochim. Biophys. Acta 581*, 228−236.
32. Frankel, L. K., and Bricker, T. M. (1995) *Biochemistry 34*, 7492−7497.
33. Xu, Q., Nelson, J., and Bricker, T. M. (1994) *Biochim. Biophys. Acta 1188*, 427−431.
34. Shutova, T., Irrgang, K.-D., Shubin, V., Klimov, V. V., and Renger, G. (1997) *Biochemistry 36*, 6350−6358.
35. Enami, I., Kamo, M., Ohta, H., Takahashi, S., Miura, T., Kusayanagi, M., Tanabe, S., Kamei, A., Motoki, A., Hirano, M., Tomo, T., and Satoh, K. (1998) *J. Biol. Chem. 273*, 4629−4634.
36. Hutchison, R., Betts, S. D., Yocum, C. F., and Barry, B. (1998) *Biochemistry 37*, 5643−5653.
37. Schweers, O., Schonbrunn-Hanebeck, E., Marx, A., and Mandelkow, E. (1994) *J. Biol. Chem. 269*, 24290−24297.

BI981305H