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Leucine 245 Is a Critical Residue for Folding and Function of the Manganese Stabilizing Protein of Photosystem II†

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ABSTRACT: In solution, Manganese Stabilizing Protein, the polypeptide which is responsible for the structural and functional integrity of the manganese cluster in photosystem II, is a natively unfolded protein with a prolate ellipsoid shape [Lydakis-Simantiris et al. (1999) *Biochemistry 38*, 404−414; Zubrzycki et al. (1998) *Biochemistry 37*, 13553−13558]. The C-terminal tripeptide of Manganese Stabilizing Protein was shown to be critical for binding to photosystem II and restoration of O_2 evolution activity [Betts et al. (1998) *Biochemistry 37*, 14230−14236]. Here, we report new biochemical, hydrodynamic, and spectroscopic data on mutants E246K, E246STOP, L245E, L245STOP, and Q244STOP. Truncation of the final dipeptide (E246STOP) or substitution of Glu246 with Lys resulted in no significant changes in secondary and tertiary structures of Manganese Stabilizing Protein as monitored by CD spectroscopy. The apparent molecular mass of the protein remained unchanged, both mutants were able to rebind to photosystem II, and both proteins reactivate O_2 evolution. Manganese Stabilizing Protein lacking the final tripeptide (L245STOP), or substitution of Glu for Leu245 dramatically modified the protein's solution structure. The apparent molecular masses of these mutants increased significantly, which might indicate unfolding of the protein in solution. This was verified by CD spectroscopy. Both mutant proteins rebound to photosystem II with lower affinities, and activation of O_2 evolution was decreased dramatically. Enhancement of these defects was observed upon removal of the final tetrapeptide (Q244STOP). These results indicate that Leu245 is essential to maintaining Manganese Stabilizing Protein's solution structure in a conformation that promotes efficient binding to photosystem II and/or for the subsequent steps that lead to enzyme activation. Based on an analysis of the properties of C-terminal mutations, a hypothesis for structural requirements for functional binding of Manganese Stabilizing Protein to photosystem II is presented. Effects of C-terminal mutations on the UV spectrum of Manganese Stabilizing Protein were also examined. Mutations that alter solution structure also affect a 293 nm absorption shoulder which is assigned to the only tryptophan residue, Trp241, in the protein, and this absorbance feature is shown to be a useful indicator of alterations to the Trp241 environment.

Oxidation of H_2O to molecular O_2, protons, and electrons is catalyzed by photosystem II (PSII)[1] in higher plants, algae, and cyanobacteria (for reviews, see refs *1* and *2*). Substrate H_2O binds on or near a redox-active tetranuclear manganese cluster, part of the so-called O_2 evolving complex (OEC), which accumulates the oxidizing equivalents necessary for H_2O oxidation during a sequence of redox reactions. These reactions, termed the S-state cycle by Joliot and Kok (*3*), are the subject of mechanistic models (*4*, *5*) that are now under investigation. The integrity and optimum function of the OEC depends on the presence of extrinsic, H_2O-soluble proteins with molecular masses of 17, 23, and 33 kDa (*6*). These proteins are involved in retention of Ca^{2+} and Cl^-, essential cofactors for H_2O oxidation reactions (*6*, *7*), at their sites of action in the OEC. Two copies of the 33 kDa protein, also known as the Manganese Stabilizing Protein (MSP), are bound to the lumenal side of PSII (*8*−*10*; for recent reviews, see refs *6*, *11*). Biochemical or genetic deletion of MSP from PSII results in increased sensitivity to photoinactivation, and loss of stability of the OEC. In the absence of high concentrations of Cl^-, two Mn ions per reaction center are lost from MSP-depleted PSII (*12*). Redox reactions of the S-state cycle are also affected by the absence of MSP. The S_2 and S_3 states exhibit increased stability, and the $S_3 \rightarrow S_4 \rightarrow S_0$ transition is slowed by a factor of 3−5 (*13*−*15*). Steady-state O_2 evolution activity is also dramatically

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[1] Abbreviations: Bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; CD, circular dichroism; Chl, chlorophyll; FT-IR, Fourier transform infrared spectroscopy; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, Manganese Stabilizing Protein; OEC, O_2 evolving complex; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; *psb*O, gene encoding precursor MSP; SDS, sodium dodecyl sulfate; SMTC, 0.4 M sucrose, 50 mM MES, 60 mM $(CH_3)_4NCl$, 20 mM $CaCl_2$; sw-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic polypeptides; UV, ultraviolet; usw-PSII, urea−NaCl-washed photosystem II membranes depleted of 33, 23, and 17 kDa extrinsic polypeptides; @, STOP codon replacing an amino acid codon.

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reduced, to approximately one-third that of the intact system (*16*). Rebinding of MSP to PSII restores normal OEC activity and Mn cluster stability at physiological Cl^- concentrations. Similarities between the effects of MSP removal and of Cl^- depletion on functional and spectroscopic properties of PSII form the basis of a proposal by Bricker and Frankel that MSP functions to regulate $Mn-Cl^-$ interactions by facilitating Cl^- retention in the vicinity of the OEC (*11*).

Biochemical removal of native MSP and subsequent binding of mutated proteins overexpressed in *E. coli* have been employed to examine the role of certain amino acid residues in binding of MSP to PSII and in reactivation of O_2 evolution (reviewed in ref *11*), and overexpressed C- and N-terminal His-tagged proteins have also been characterized (*17*). These proteins reconstitute PSII activity to normal levels, although C-terminal His-tagged MSP showed a sigmoidal binding/reactivation curve (*17*). Information on the role of the N-terminus of MSP was provided by Eaton-Rye and Murata, who removed the first 16−18 N-terminal amino acids by proteolysis and showed that the truncated protein was unable to bind to PSII (*18*). The role of the MSP C-terminus in binding and restoration of PSII activity was examined using truncation mutants lacking the terminal di-, tri-, and tetrapeptides (*19*). This study revealed that the terminal dipeptide, -EQ, is not necessary for MSP function, but further truncations resulted in dramatic decreases in binding and reactivation capacities of the mutated MSPs. Intramolecular cross-linking studies by Enami and coworkers showed that C-terminal amino acid residues of MSP are in van der Waals contact distance from residues near the N-terminus when MSP is in solution and that this structural conformation changes when MSP is bound to PSII (*20*, *21*). Conformational changes in MSP upon binding to PSII were also detected by an isotope-editing technique using FTIR spectroscopy (*22*).

In solution, MSP exhibits an unusual behavior that is detected by SDS−polyacrylamide gel electrophoresis and by gel filtration. Although the protein's molecular mass from the DNA sequence is 26 540 Da (*23*), MSP migrates at about 33 kDa on SDS gels (*24*), and is even larger (41 kDa) according to its elution behavior on gel filtration columns (*19*, *25*). Manganese Stabilizing Protein is acidic [pI = 5.2 (*24*)]. Simulations of its far-UV CD spectrum at room temperature predict a secondary structure consisting predominantly (∼50%) of turns and random coil, with a small (7−10%) α-helical and a relatively larger (∼35%) β-sheet component (*25*−*27*). Last, MSP is thermostable; solution structure and functional properties are recovered after extensive (2 h) incubation at high temperatures (90 °C) (*25*). Taken together, these properties (acidic p*I*, high content of turns and random coil, anomalous behavior in gel electrophoresis and gel filtration experiments, thermostability) indicate that MSP belongs to a group of proteins that are said to be "natively unfolded" (*25*).

In this communication, site-directed mutagenesis has been combined with biochemical and spectroscopic methods to identify amino acids at the C-terminus of MSP that are critical for binding to PSII and reactivation of O_2 evolution. Experiments are presented which correlate the biochemical behavior of MSP mutants with accompanying structural changes induced by the mutations. It is also shown that a 293 nm UV absorption band, assigned to the sole tryptophan residue in MSP, can be used to monitor changes in the environment of the protein's C-terminus.

MATERIALS AND METHODS

Isolation of PSII Membranes. PSII membranes were isolated from market spinach by the method of Berthold et al. (*28*) with modifications (*29*). Intact PSII membranes were frozen in liquid N_2 immediately after preparation and kept at −70 °C until use. Extraction of 17 and 23 kDa polypeptides was carried out as described in ref *30*, and MSP was extracted as described in ref *32*. The MSP-depleted PSII samples were stored at −70 °C in 0.4 M sucrose, 200 mM NaCl, 50 mM MES (pH 6.0). Extracted MSP was further purified by anion exchange chromatography (see below) and used as control along with recombinant wild-type MSP. Oxygen evolution activity was measured using a Clark-type electrode under conditions described in ref *25*. Typical rates for intact PSII, NaCl-washed PSII, and urea-washed PSII were 500−600, 400−500, and about 200 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}, respectively.

Mutation of psbO Gene and Transformation of E. coli Cells. For construction of the L245E mutation, the oligonucleotide 5′-TGAATTCCTTTTATTGCTCCTCTTGTG-CATACC-3′, based on the antisense strand at the C-terminal encoding region of MSP (the underlined triplet indicates the change to glutamic acid), was designed and synthesized (Life Technologies, Gibco BRL custom primers). This oligonucleotide contains one *Eco*RI endonuclease restriction site three nucleotides after the stop codon of *psb*O. One more oligonucleotide (5′-CCATATGGCAGCTTCATTACAAGCATC-3′), identical to the sense strand of the *psb*O gene at the 5′ encoding region, was synthesized. The coding region was amplified by polymerase chain reaction using the oligonucleotides above as primers. Polymerase chain reaction products were cut with *Eco*RI and *Bam*HI, and the 0.3 kb *Eco*RI−*Bam*HI fragment was gel-purified and cloned into *Eco*RI- and *Bam*HI-cut pET 8c vector containing wild-type *psb*O (*31*, *32*). The resulting pET vector containing *psb*O-(L245E) was inserted into BL21(DE3)pLysS *E. coli* competent cells. Q244@, L245@, E246@, and E246K mutants were constructed as described in ref *19*. All mutations were confirmed by DNA sequencing, and the integrity of overexpressed MSP was examined by Edman analysis of the first five N-terminal amino acids.

Isolation and Purification of Native and Recombinant MSP. Native MSP extracted by incubation of NaCl-washed PSII in 2.6 M urea/200 mM NaCl refolded slowly by dialysis against 100 mM tris(hydroxymethyl)aminomethane (pH 8.0), 10 mM NaCl for 5 h. Then, a second dialysis step was carried out against 50 mM MES (pH 6.0), 10 mM NaCl for 5 h. The dialyzed protein was then diluted with an equal volume of 50 mM MES (pH 6.0), 10 mM NaCl, 5% betaine (buffer MES-A), centrifuged (40000*g*, 30 min), and loaded onto a Resource Q column (Pharmacia Biotech) equilibrated with MES-A. A step gradient (30 mM, 150 mM, and 500 mM NaCl) was applied, and pure native MSP was eluted at the intermediate NaCl concentration. Recombinant MSP was overexpressed by isopropyl β-D-thiogalactopyranoside induction of transformed *E. coli* cells as described in ref *30*. Inclusion bodies containing mature MSP were isolated by sonication of bacterial cells, centrifugation, and subsequent

washing with dodecyl maltoside as described in ref *32*. Purified inclusion bodies were then solubilized by 3.0 M urea, and the unfolded MSP was loaded on a Resource Q column equilibrated with 3 M urea, 20 mM Bis-tris (pH 6.4), 5 mM NaCl, 5% betaine (Urea-A). A linear gradient (5 mM to 250 mM NaCl) was applied, and recombinant MSP was eluted at about 120 mM NaCl. A second chromatographic step was then applied to further purify MSP. The conditions used for this step varied to accommodate the elution behavior of the different MSP species. Recombinant wild-type MSP was refolded after the first FPLC step by dialysis against 50 mM MES (pH 6.0), 10 mM NaCl, loaded onto a Resource Q column equilibrated with MES-A buffer, and eluted by a step gradient as described above for native MSP. For the mutant proteins, refolding by dialysis was omitted, and a step gradient (30, 60, 200, and 500 mM NaCl) was applied after reloading the protein onto a Resource Q column equilibrated with Urea-A buffer. Pure MSP was eluted at 200 mM NaCl. Then, refolding was carried out as described above. Sample purification was monitored by SDS−PAGE using the Neville buffer system (*33*). Protein concentrations were estimated spectrophotometrically at 276 nm using an extinction coefficient of 16 mM^{-1} cm^{-1} (*8*).

Rebinding and Reactivation Analyses. Native, recombinant wild-type and mutant MSPs were mixed with urea−salt-washed PSII (0.2 mg of Chl/mL) at ratios from 0.5 to 10 mol of MSP/mol of PSII, and the reconstitution mixture was incubated at room temperature, in the dark, for 1 h as described in ref *30*. Then, the reconstituted samples were transferred to ice, and O_2 evolution activity was measured with a Clark-type electrode as described in ref *25*. The final concentration of the sample for the O_2 evolution activity measurements was 10 μg of Chl/mL in 0.4 M sucrose, 50 mM MES (pH 6.0), 60 mM $(CH_3)_4NCl$, 20 mM $CaCl_2$ (SMTC) with 600 μM 2,6-dichloro-*p*-benzoquinone as the electron acceptor. Two types of experiments were carried out: estimation of the rates of O_2 evolution stimulated by saturating white light and measurements of the total O_2 yield in 4 min under 80% light saturation. The former experiment monitors the rate of PSII turnover whereas the latter monitors the stability of the reactivated system. To quantify the binding ability of MSP wild-type and mutants, reconstituted samples were washed by centrifugation (40000*g*, 30 min) to remove the unbound MSP and resuspended in SMTC at a final concentration of 1 mg of Chl/mL. Coomassie-stained SDS gels of these samples were analyzed by Sigmagel (Jandel Scientific). The 47 kDa band was used as an internal standard to correct for inconsistencies in sample loading and electrophoresis.

CD and Optical Spectroscopy. For CD spectroscopy, an AVIV 62DS instrument was used as described in ref *25*. Before data acquisition, the instrument was calibrated with 1 mg/mL (+)-10-camphorsulfonic acid (*34*). For both far- and near-UV CD, previously refolded MSP samples were dialyzed against 10 mM KH_2PO_4, pH 6.0, for 5 h. For far-UV CD, MSP samples were adjusted to 10 μM and transferred to a 1 mm wide quartz cuvette. A total of 20 scans (250−184 nm) were averaged, and the resulting spectrum was corrected for the base line (average of 20 scans of 10 mM KH_2PO_4, pH 6.0 solution). Circular dichroism spectra were analyzed by several programs [SELCON, CONTIN (*35*−*37*)], kindly provided by Prof. Norma Greenfield (Neuroscience and Cell Biology Department, Robert Wood Medical School, Piscataway, NJ). More experimental conditions are provided in the figure legends. For the near-UV CD, MSP samples were transferred to 1 cm wide cuvettes, and 10 scans (330−250 nm) were averaged and corrected for the base line. The spectra were then normalized to the spectrum of the most concentrated sample. Optical spectroscopy was carried out at room temperature on an OLIS-modified Cary-17 instrument. All samples were in 10 mM KH_2PO_4, pH 6.0, except for unfolded wild-type MSP which was transferred by dialysis to 3 M urea, 50 mM MES, pH 6.0, 50 mM NaCl. Other experimental conditions are given in the figure legends.

Size-Exclusion Chromatography. For size-exclusion chromatography, a Superose-12 column (Pharmacia HR 10/30) attached on a Pharmacia FPLC system was equilibrated with 20 mM Bis-tris (pH 6.4), 150 mM NaCl. Blue dextran was used to determine the void volume of the column. The elution volumes of bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa) were determined and used to construct a calibration curve.

RESULTS

Leu245 Is Critical for MSP Binding and Function. Truncation of the C-terminal tripeptide, -LEQ, caused a reduction in the shortened protein's ability to rebind to PSII and to restore activity, and removal of the C-terminal tetrapeptide (-QLEQ) magnified these effects (*19*). However, the question of whether loss of a specific amino acid, rather than removal of the entire tripeptide, was responsible for altered MSP activity has not been addressed. To accomplish this, biochemical and structural properties of native and wild-type recombinant MSP were compared with those of (1) mutant proteins lacking the C-terminal di-, tri-, or tetrapeptide and with (2) mutant proteins in which specific residues were changed. In these experiments, MSP-depleted PSII was reconstituted with mutated and wild-type proteins, binding capacities were determined, and activity restoration was assayed. Effects of mutations on MSP secondary structure were examined by far-UV CD spectroscopy, and changes in tertiary structure were probed by near-UV CD, size-exclusion chromatography, and optical spectroscopy. In the first set of experiments, residue Leu245 was changed to Glu. This residue was targeted, since its removal in tripeptide truncation experiments was shown to produce a substantial alternation to hydrodynamic and activity properties of the resulting MSP mutant (*19*).

Figure 1A presents an SDS gel that summarizes results from a L245E-PSII rebinding experiment. To exclude the possibility that rebinding defects were due to damaged urea−salt-washed PSII samples, reconstitution was also performed with native and recombinant wild-type MSP (3−5 mol of MSP/mol of PSII). A rebinding efficiency of 95−110%, as compared to salt-washed PSII, was consistently observed (data not shown), attesting to the integrity of the urea−salt-washed material. Figure 1B shows results of densitometric analyses of rebinding experiments with L245E and for a series of MSP mutants including Q244@, L245@, E246@, and E246K. These data show that mutations or truncations that include Leu245 cause dramatic changes in MSP binding.

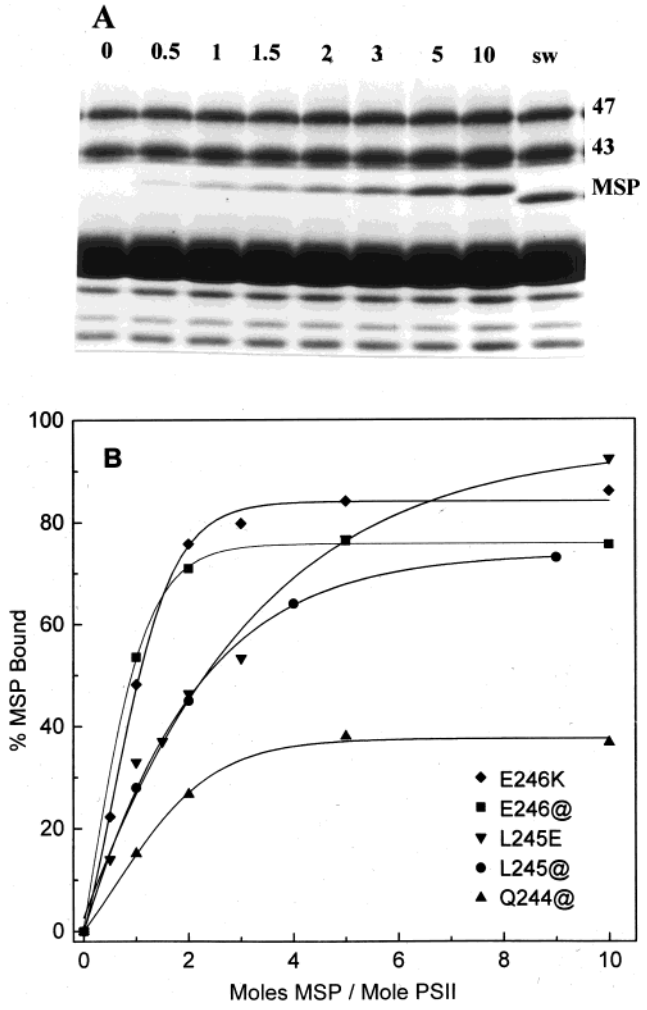

FIGURE 1: (A) Coomassie-stained SDS−PAGE of usw-PSII samples reconstituted with L245E MSP. The mol of MSP/mol of PSII used for reconstitution is indicated at the top of the lanes; 30 μg of Chl was loaded in each lane. (B) Binding curves for usw-PSII reconstituted with C-terminal MSP mutants. SDS−PAGE gels of reconstituted samples were analyzed by densitometry, and the binding efficiency of MSP mutants was expressed as the percentage of MSP in sw-PSII (100% control). The 47 kDa band was used as an internal standard.

Deletion of -LEQ (L245@) or substitution of Leu245 with Glu (L245E) reduces the affinity of MSP for PSII, as is evident by the lower slopes of the corresponding binding curves in Figure 1B. The mutant L245E shows a higher efficiency of binding to PSII (90% at 10 mol of MSP/mol of PSII) as compared to the truncation mutant L245@ (70−75%, see also ref *19*). By way of comparison, removal of the sequence -QLEQ (Q244@) causes an even more dramatic reduction of MSP binding capacity, as evidenced by the Q244@ binding curve shown in Figure 1B (~35% of control binding at 10 mol of MSP/mol of PSII; see also ref *19*). The data in Figure 1B also reinforce the conclusion (*19*) that the C-terminal dipeptide (-EQ) is not required for binding of MSP to PSII. Removal of the dipeptide (E246@) or substitution of Glu246 with Lys (E246K) causes only minor changes in the binding efficiency of MSP.

The ability of the mutations shown in Figure 1 to restore PSII activity is summarized by the data of Figure 2; total O_2 production during a 4 min nonsaturating illumination of reconstituted samples is presented as a percentage of the control (sw-PSII, 100%). The high O_2 yields for E246@ and

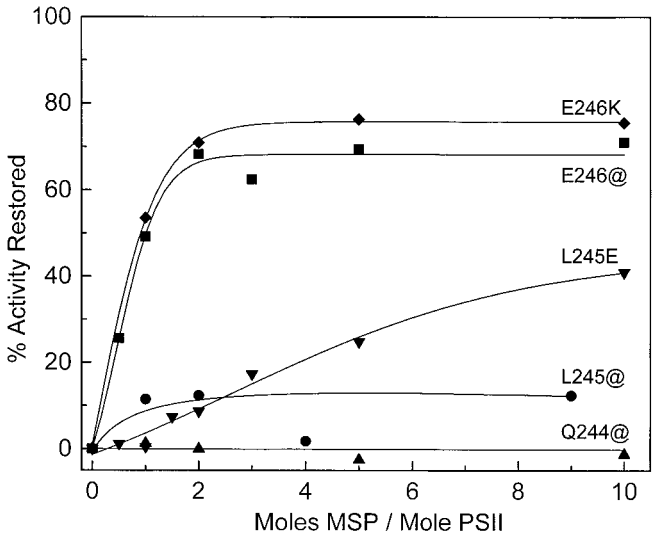

FIGURE 2: Recovery of O_2 evolution by usw-PSII samples reconstituted with MSP mutants. The total amount of O_2 produced during 4 min of nonsaturating illumination was expressed as a percentage of the amount of O_2 produced by a salt-washed PSII sample under the same conditions (28 μmol of O_2/mg of Chl, 100% control). The residual activity of usw-PSII was subtracted from the activities of the reconstituted samples. Each data point is the average of three measurements; the error for each data point is approximately ±5%.

Table 1: Oxygen Evolution Activity of PSII Reconstituted with Wild-Type and Mutant MSPs[a]

mutants	activity (% control)	mutants	activity (% control)
wild type	80−85 (2)	E246@	70 (2)
E246K	75 (2)	L245@	8 (2−3)
L245E	36 (10)	Q244@	0 (10)

[a] The 100% activity (sw-washed PSII) was ~500 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Numbers in parentheses designate mol of MSP/mol of PSII for the reconstituted samples assayed.

E246K mutants support the earlier observation that the C-terminal dipeptide -EQ is not essential for MSP function. Saturation of O_2 yield occurred at 2 mol of MSP/mol of PSII for both mutants, with the E246K-reconstituted sample giving slightly higher activity. On the other hand, substitution of Leu245 with Glu produced a severe reduction of O_2 yield in the reconstituted enzyme. At 2 mol of MSP/mol of PSII, only 8−10% of activity was restored even though 50% of the control level of MSP was bound; activity increased to about 40% of the control at 10 mol of MSP/mol of PSII. Removal of the C-terminal tripeptide (-LEQ) resulted in even more pronounced damage to MSP's ability to reactivate O_2 production. Only 10% of control activity was restored at 9−10 mol of MSP/mol of PSII in samples that showed 70% of control rebinding (Figure 1; see also ref *19*). Removal of the tetrapeptide -QLEQ resulted in complete loss of enzyme activity (Figure 2). Initial rates of O_2 production were also assayed for all reconstituted PSII samples under saturating illumination. Rates of O_2 evolution activity obtained in PSII samples reconstituted with saturating amounts of MSP are shown in Table 1; in general, the activity vs MSP concentration curves obtained from these experiments (data not shown) closely paralleled the corresponding O_2 yield curves presented in Figure 2.

Modification of Leu245 Causes Unfolding of MSP. When the effects of C-terminal mutations on MSP secondary

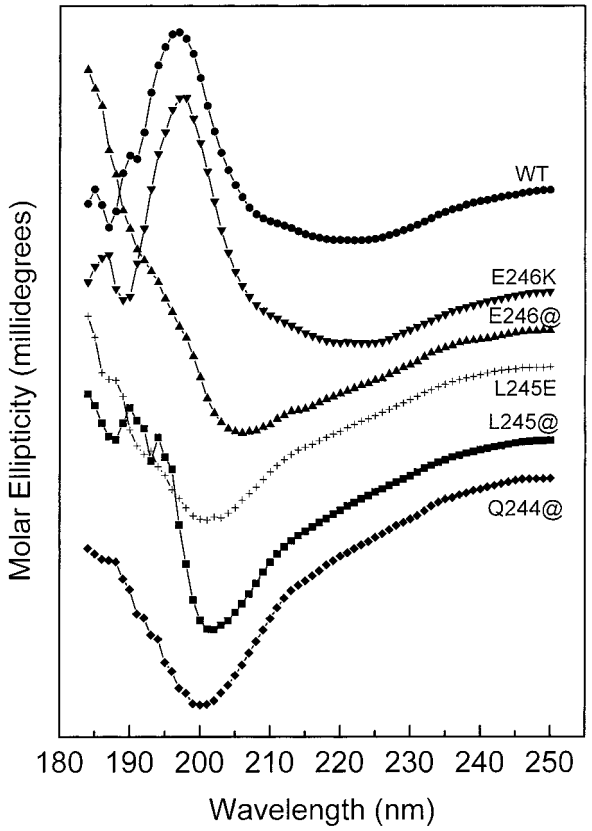

FIGURE 3: Far-UV CD spectra (protein solution minus buffer) of C-terminal MSP mutants (10 μM protein in 10 mM KH_2PO_4, pH 6.0). Each spectrum is the average of 20 scans. Experimental conditions: scan width, 250−184 nm; time constant, 1 s; bandwidth, 1.5 nm; temperature, 25 °C; path length, 1 mm. Each spectrum was smoothed by the adjacent averaging method (mean value of 2 adjacent points as the new data point) after analysis of the spectrum for prediction of the structural contents.

structure were examined by far-UV CD spectroscopy, the results shown in Figure 3 were obtained. These spectra can be divided into three sets: those showing normal MSP secondary structure (wild type and E246K); spectra of modified secondary structure (E246@); and spectra indicative of unfolded proteins (L245E, L245@, Q244@). By combining these data with the results in Figures 1 and 2, tentative correlations between MSP secondary structure in solution and its ability to bind to PSII and reactivate the O_2 evolving reaction can be made. Mutants retaining minimal secondary structure (E246K and E246@) are able to bind and to reactivate PSII with efficiencies comparable to wild-type MSP, while mutants with far-UV CD spectra resembling thermally unfolded MSP (*25*) either bind to PSII with considerably lower affinity and minimal restoration of activity (L245E and L245@) or bind very weakly and are completely unable to reactivate PSII (Q244@). It must be emphasized that binding, as determined by SDS−PAGE, could also represent nonphysiological, unspecific associations of MSP with usw-PSII if this adventitious association is strong enough to withstand removal from PSII by the washing steps before gel electrophoresis as described under Materials and Methods.

Simulations of the CD spectra in Figure 3 using SELCON and CONTIN software (*35*, *36*) resulted in secondary structure predictions shown in Table 2. Wild-type secondary structure prediction agrees well with previously reported

Table 2: Predicted Secondary Structure Components of MSP Based on Far-UV CD Spectra

protein	α-helix	β-sheet	turns/random coil	total
wild type	7	44	49	100
E246K	5	44	47	96
E246@	11	33	56	100

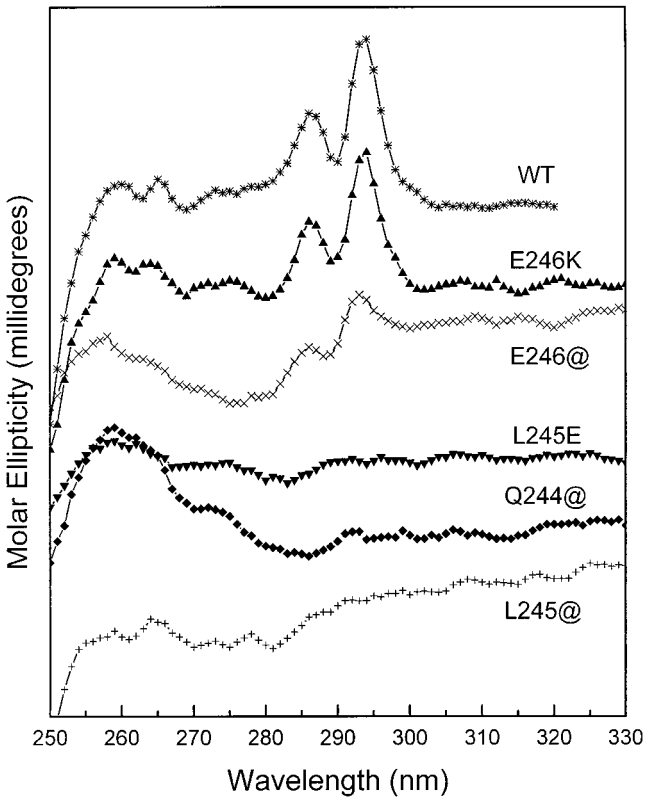

FIGURE 4: Near-UV CD difference spectra of C-terminal MSP mutants. Each spectrum is the average of 10 scans. Scan width, 250−320 (WT) or 330 nm; path length, 1 cm. Other experimental conditions as in Figure 3. Spectra are normalized to correct for concentration differences.

values (*26*, *27*). For E246K, the secondary structure prediction closely resembles wild-type MSP whereas E246@ is predicted to contain an apparent increase of α-helix and random coil at the expense of β-sheet. Due to lack of structural data from unfolded proteins in the databases used for structure predictions, L245E, L245@, and Q244@ CD spectra could not be analyzed in a consistent, reliable way. However, very little secondary structure can be expected to exist in these mutants since their CD spectra are very similar to those of thermally unfolded wild-type MSP, and of other unfolded proteins from a variety of sources (*25*, *34*).

Near-UV CD spectroscopy provides information about the tertiary structure of a protein (*38*). Aromatic amino acids (tyrosine, tryptophan, and phenylalanine) absorb at characteristic wavelengths, and their CD spectra depend on the protein environment. Thus, changes in tertiary structure affecting the environment of these amino acids will, in general, change the corresponding near-UV CD spectra. Figure 4 shows the near-UV CD spectra of wild-type MSP and C-terminal mutants. The CD spectrum of E246K again resembles the wild-type CD spectrum; both spectra show the characteristic peaks assigned to tryptophan (294 nm) and tyrosine (285 nm). The E246@ spectrum shows the same

Table 3: Apparent Molecular Masses for MSP Wild Type and Mutants Based on Size-Exclusion Chromatography

mutants	apparent molecular mass (kDa)	reference
wild type	41	*19*, *25*, this work
E246K	41	this work
L245E	62	this work
E246@	42	*19*, this work
L245@	52	*19*, this work
Q244@	58	*19*, this work

peaks, but has a distorted appearance when compared to the spectrum of wild-type MSP. Near-UV CD spectra of L245E, L245@, and Q244@ do not contain any peaks, and are very similar to the spectrum obtained for wild-type MSP at 90 °C (*25*).

The data of Figure 4 indicate that the correlation between MSP secondary structure content and function would appear to be valid for tertiary structure as well. To see if this is so, structural predictions from near-UV CD spectroscopy were tested by size-exclusion chromatography. Previous research (*19*) showed that removal of the C-terminal dipeptide did not affect the apparent molecular mass of MSP, but further truncations caused apparent unfolding of the protein and, consequently, higher apparent molecular masses. It could not be determined at that time whether the increase in the apparent size of MSP was due to the loss of several amino acids, or to the loss of a critical amino acid residue. Therefore, effects of mutations at the C-terminus of MSP on the apparent mass of the protein were examined. Table 3 shows that E246K MSP exhibits the same apparent molecular mass as wild-type protein, while L245E yields an apparent molecular mass of 62 kDa. It is interesting that this value is even greater than the apparent mass of L245@.

UV absorption spectroscopy can be employed to monitor structural changes in proteins (*39*), owing to the sensitivity of aromatic amino acids to changes in the local residue environment (i.e., exposure of hydrophobic regions to solvent). These changes can take the form of blue shifts of spectra and/or changes in the amplitude of characteristic absorption peaks (*39*). Figure 5A presents UV spectra of wild-type MSP and of the mutants described above. The peaks contributing to the MSP UV spectrum are not well resolved except for the characteristic shoulder at 293 nm which arises from tryptophan absorption. Unfolding of wild-type MSP with urea results in loss of this shoulder, indicating that the hydrophobicity of the environment of the single MSP tryptophan residue, Trp241 (40), is altered by urea exposure. Comparison of the UV spectrum of native wild-type MSP with the spectra of mutant MSPs (Figure 5A) shows that the E246K mutation does not affect the Trp241 shoulder, whereas the E246@ mutation causes a slight decrease in amplitude and a small shift (about 1.5 nm) to the blue. However, mutation/truncation of the C-terminal tripeptide or tetrapeptide results in complete disappearance of the 293 nm shoulder. The difference spectrum of wild-type minus Q244@ is shown in Figure 5B. Similar difference spectra can be obtained by subtraction of the L245E and L245@ spectra from a wild-type MSP spectrum (data not shown). The major features of this difference spectrum are a positive peak at 293 nm and a shoulder at about 287 nm. The peak at 293 nm originates from Trp241 whereas the shoulder at 287 nm is due to changes in the local environment of one or more of the tyrosine residues in MSP sequence (*23*, *40*). The residue adjacent to Trp241, Tyr242, may be responsible for the 287 nm shoulder, but contributions from other tyrosine residues to this spectral feature cannot be excluded.

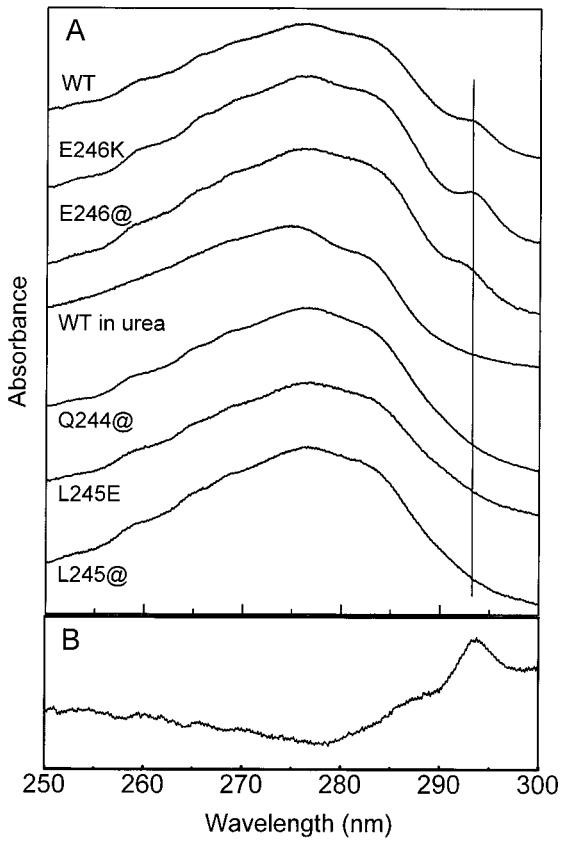

FIGURE 5: (A) UV spectra of C-terminal MSP mutants (10 μM protein in 10 mM KH_2PO_4, pH 6.0). Experimental conditions: scan width, 240−310 nm; room temperature; path length, 1 cm. The vertical line is used to mark the position of 293 nm. Spectra are normalized to correct for concentration differences. (B) Difference spectrum of wild-type MSP minus Q244@.

DISCUSSION

A prior examination of the properties of MSP mutants with truncated C-terminal di-, tri-, and tetrapeptides showed that although the terminal dipeptide (-EQ) of MSP was unnecessary for binding and restoration of PSII function, removal of the C-terminal tri- or tetrapeptide decreased MSP's ability to bind to PSII and to restore O_2 evolution activity (*19*). These experiments defined the requirements for C-terminal peptides, but the essential role(s) of particular amino acids in these peptides was (were) not established. To resolve this question, additional mutations were designed at the MSP C-terminus in which Leu245 was replaced by Glu, and Glu246 was replaced by Lys, without affecting the total length of MSP.

The data in Figures 1B and 2 show that the biochemical behaviors of E246@ and of E246K are similar to that of wild-type MSP with respect to binding affinity and activity restoration. The CD spectra of these mutants reveal a change in secondary structure only for E246@, where a gain of α-helix and random coil is predicted at the expense of β-sheet (Table 2). Reconstitution of PSII with this mutant is temperature-sensitive (*19*), and its CD spectrum is very similar to that of the V235A MSP mutant, whose reconstitu-

tion activity is likewise temperature-sensitive (*41*). The near-UV CD spectra of E246K and E246@, presented in Figure 4, also show either no change (E246K) or small changes (E246@) in overall tertiary structure, and size-exclusion chromatography reveals no significant differences in the apparent molecular masses of these proteins (Table 3). Taken together, these results suggest that Glu246 is unnecessary for MSP's structure and function. Enami et al. (*20, 21*) have shown that intramolecular cross-linking occurs between Glu246 and Lys48 when MSP in solution was reacted with EDC, whereas PSII-bound MSP yields a Glu246−Lys190 cross-link (*20, 21*). Our results for residue 246 of the spinach protein show that interactions between Glu and other amino acids of MSP, either in solution or bound to PSII, can be abolished without affecting the protein's structure and function.

In contrast, Leu at position 245 is critical for MSP's solution structure and for its ability to bind to PSII; removal of the C-terminal tripeptide, or substitution of Glu for Leu, results in a substantial disruption of MSP secondary and tertiary structure, as is evidenced by CD spectra (Figures 3 and 4) and by increases in the apparent molecular mass (Table 3). Size-exclusion chromatography in 3 M urea was necessary to increase the apparent molecular mass of wild-type MSP to that of L245@ (*30*), and conversion of Leu to Glu had an even more dramatic effect on the apparent mass. These results suggest that 3 M urea may be insufficient to unfold MSP completely (see also Figure 4 in ref *30*). Results from SDS−PAGE in 4.7 M urea show that L245E migrates more slowly than wild-type MSP (Figure 1A), whereas L245@ migrates slightly faster (data not shown, but see ref *19*).

The structural changes in MSP due to mutations at Leu245 cause a significant decrease in PSII binding affinity (Figure 1B). L245E binding saturates (about 90% of wild type) at a ratio of >10 mol of MSP/mol of PSII whereas L245@ binding saturates at about 70% of wild-type levels at the same MSP/PSII ratio. These results may reflect both specific and adventitious binding of MSP to PSII, such as has been reported for wild-type recombinant MSP after treatment at high temperatures (*25*). If so, reconstitution data on Leu245 mutants might be interpreted in two ways. First, the protein may bind specifically to its native sites on PSII, at a lower affinity than wild type, but cannot refold properly. Restoration of O_2 evolution would occur at moderate (L245E) or very low (L245@) levels. Alternatively, Leu245 mutants may bind to both native and adventitious sites on PSII, with the adventitious sites having affinities comparable to those of the native sites. Residual O_2 evolution activity would be restored only to those centers that have MSP bound to native sites. It is interesting that the slopes of the binding curves of L245E and L245@ are the same, but the restoration of O_2 evolution activity by L245E is higher than for L245@. Although neither hypothesis can be eliminated at present, both of them are consistent with a proposed two-step mechanism for MSP function (*42*). According to this model, MSP first binds to PSII, and then, in a second step, conformational changes occur which result in a stable, functional MSP−PSII interaction. If the first hypothesis is correct, one would expect mutations at Leu245 to affect the second step in this mechanism, whereas if the second hypothesis is correct, the first step will be affected. The important conclusion from the work presented here is that MSP function is impaired if Leu245 is absent, and because truncation of the C-terminal tetrapeptide (-QLEQ) results in an enhancement of the effects involving Leu245, Gln244 may also be important for MSP function. Work is in progress to resolve this issue.

Table 4: Alignment of C-Terminal MSP Sequence: Conserved Residues Are Indicated by a Dash, and the Residue at Position 245 Is Indicated in Italic Boldface Type

	species	C-terminal sequence
eukaryotes	*S. oleracea*	V K I E G V W Y A Q L E Q
	S. tuberosum	- - - Q - I - - - - - -
	P. sativum	- - - Q - - - - - - - -
	T. aesitivum	- - - Q - - - - - - - -
	A. thaliana	- - - Q - - - - - - - -
	C. reinhardtii	- - V T - L - - - - - K
	E. gracilis	I - T S - - - - - - ***I*** S P S K
prokaryotes	*S. elongatus*	- - - Q - - F - - S ***I*** - P A
	A. nidulans	- - L V - Q F - G R ***I*** - P A D A
	Synechocystis PCC6803	- - V R - I F - G R ***V*** D T D V
	Anabaena PCC7120	- - - R - I F - - R ***V*** E

A consequence of disruption of the tertiary structure of MSP by C-terminal mutations is the decrease and/or shift to the blue of the 293 nm UV band assigned to Trp241. In wild-type MSP, this band is relatively well resolved, but disappears upon exposure of the protein to urea (Figure 5A). Mutation or truncation at Glu246 does not substantially affect the 293 nm absorption band, except for a small shift (about 1.5 nm) to the blue caused by mutation E246@. This blue shift is consistent with the small change in the near-UV CD spectrum of this mutant, as compared to E246K and to wild type (Figure 4). However, mutation or truncation at Leu245 or Gln244 produces a complete loss of the 293 nm band. These results suggest that Trp241 in wild-type MSP resides in a hydrophobic environment that is disrupted by modifications of Leu245 and Gln244. Thus, a clear correlation exists between the tertiary structure of MSP and its UV absorption at 293 nm. Changes in the 293 nm band also correlate with the biochemical behavior of MSP mutants. The absorption band is resolved only in those MSP species (wild type, E246K, E246@) that can efficiently rebind to PSII and reactivate O_2 evolution. The model for MSP solution structure of Bricker and Frankel (*11*) proposes that in solution MSP contains 11 β-strands which form mostly antiparallel β-sheets. In this model, Trp241 resides in the final β-strand which also contains Leu245 as its final residue. If this strand is part of a hydrophobic sequence in MSP that is critical for the solution structure necessary for specific binding to PSII, then Leu245 may be required to maintain this structure. Mutations or truncations affecting this residue would cause structural changes, possibly by exposing this domain to the solvent, as evidenced by changes in the 293 nm UV absorption band reported here. The C-terminal dipeptide is not predicted to participate in these hydrophobic interactions, and this is consistent with failure of mutations at this position to affect the biochemical behavior of the protein.

The C-terminal sequences of several MSP species are aligned in Table 4. Leu245 is conserved in all eukaryotes [with the exception of *E. gracilis*, where a conservative substitution (Ile) has been made] and is conservatively substituted (Ile or Val) in prokaryotes. Several other hydrophobic residues of the C-terminus of MSP (Val235, Ile237,

Gly239, Val240, Trp241, Tyr242, and Ala243) are also conserved or conservatively substituted. This suggests that these residues form a hydrophobic strand which could interact with residues near the MSP N-terminus to produce the prolate ellipsoid shape of MSP that has been revealed by hydrodynamic studies (*43*). Our data on Leu245 support the idea that this ellipsoid conformation is stabilized in solution by weak hydrophobic interactions. Addition of a single charge (as in the case of L245E) or deletion of Leu245 disrupts that solution conformation. In light of the conservation of amino acid sequence identity, it is possible that any mutation within the conservative hydrophobic residues of the C-terminal β-strand of MSP will cause severe structural and functional changes. It is also possible that the ellipsoid conformation of MSP is relatively easy to disrupt because this conformation must be altered as part of the mechanism for assembly of MSP into PSII (*21*, *22*). The necessity for a flexible solution conformation, which would facilitate protein−protein interactions, has been proposed to be the origin of the unusual behavior of natively unfolded proteins (*44*).

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REFERENCES

1. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
2. Nugent, J. H. A. (1996) *Eur. J. Biochem. 237*, 519−531.
3. Kok, B., Forbush, B., and McGloin, M. (1970) *Photochem. Photobiol. 11*, 457−475.
4. Hoganson, C. W., Lydakis-Simantiris, N., Tang, X.-S., Tommos, C., Warncke, K., Babcock, G. T., Diner, B. A., McCracken, J., and Styring, S. (1995) *Photosynth. Res. 46*, 177−184.
5. Britt, R. D. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 137−164, Kluwer Academic Publishers, Dordrecht, The Netherlands.
6. Seidler, A. (1996) *Biochim. Biophys. Acta 1277*, 35−60.
7. Yocum, C. F. (1991) *Biochim. Biophys. Acta 1059*, 1−15.
8. Xu, Q., and Bricker, T. M. (1992) *J. Biol. Chem. 267*, 25816−25821.
9. Leuschner, C., and Bricker, T. M. (1996) *Biochemistry 35*, 4551−4557.
10. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1997) *Biochemistry 36*, 4047−4053.
11. Bricker, T. M., and Frankel, L. K. (1998) *Photosynth. Res. 56*, 157−173.
12. Miyao, M., and Murata, N. (1984) *FEBS Lett. 170*, 350−354.
13. Miyao, M., Murata, N., Lavorel, J., Maison-Peteri, B., Boussac, A., and Etienne A.-L. (1987) *Biochim. Biophys. Acta 890*, 151−159.
14. Burnap, U. L., Shen, J.-R., Jursinic, P. A., Inoue, Y., and Sherman, L. A. (1992) *Biochemistry 31*, 7404−7410.
15. Razeghifard, M. R., Wydrzynski, T., Pace, R. J., and Burnap, R. L. (1997) *Biochemistry 36*, 14474−14478.
16. Bricker, T. M. (1992) *Biochemistry 31*, 4623−4628.
17. Seidler, A. (1994) *Protein Eng. 7*, 1277−1280.
18. Eaton-Rye J. J., and Murata, N. (1989) *Biochim. Biophys. Acta 977*, 219−226.
19. Betts, S. D., Lydakis-Simantiris, N., Ross, J. R., and Yocum, C. F. (1998) *Biochemistry 37*, 14230−14236.
20. Miura, T., Shen, J.-R., Takahashi, S., Kamo, M., Nakamura, E., Ohta, H., Kamei, A., Inoue, Y., Domae, N., Takio, K., Nakazato, K., Inoue, Y., and Enami, I. (1997) *J. Biol. Chem. 272*, 3788−3798.
21. 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.
22. Hutchison, R. S., Betts, S. D., Yocum, C. F., and Barry, B. A. (1998) *Biochemistry 37*, 5643−5653.
23. Tyagi, A., Hermans, J., Steppuhn, J., Jansson, Ch., Vater, F., and Herrmann, R. G. (1987) *Mol. Gen. Genet. 207*, 288−293.
24. Kuwabara, T., and Murata, N. (1979) *Biochim. Biophys. Acta 581*, 228−236.
25. Lydakis-Simantiris, N., Hutchison, R. S., Betts, S. D., Barry, B. A., and Yocum, C. F. (1999) *Biochemistry 38*, 404−414.
26. Xu, Q., Nelson, J., and Bricker, T. M. (1994) *Biochim. Biophys. Acta 1188*, 427−431.
27. Shutova, T., Irrgang, K.-D., Shubin, V., Klimov, V. V., and Renger, G. (1997) *Biochemistry 36*, 6350−6358.
28. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
29. Ghanotakis, D. F., Topper, J., Babcock, G. T., and Yocum, C. F. (1984) *Biochim. Biophys. Acta 767*, 524−531.
30. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1996) *Biochim. Biophys. Acta 1274*, 135−142.
31. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
32. Betts, S. D., Hachigian, T. M., Pichersky, E., and Yocum, C. F. (1994) *Plant Mol. Biol. 26*, 117−130.
33. 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, Amsterdam, The Netherlands.
34. Johnson, W. C., Jr. (1990) *Proteins: Struct., Funct., Genet. 7*, 205−214.
35. Provencher, S. W., and Glöckner, J. (1981) *Biochemistry 20*, 33−37.
36. Sreerama, N., and Woody, R. W. (1993) *Anal. Biochem. 209*, 32−44.
37. Greenfield, N. J. (1996) *Anal. Biochem. 235*, 1−10.
38. Kelly, S. M., and Price, N, C., (1997) *Biochim. Biophys. Acta 1338*, 161−185.
39. Schmid, F. X. (1997) in *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) 2nd ed., pp 261−267, Oxford University Press Inc., New York.
40. Oh-oka, H., Tanaka, S., Wada, K., Kuwabara, T., and Murata, N. (1986) *FEBS Lett. 197*, 63−66.
41. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1996) *Biochemistry 35*, 6302−6307.
42. Lydakis-Simantiris, N., Hutchison, R. S., Betts, S. D., Barry, B. A., and Yocum, C. F. (1998) in *Photosynthesis: Mechanisms and Effects* (Garab, G., Ed.) pp 1253−1258, Kluwer Academic Publishers, Dordrecht, The Netherlands.
43. Zubrzycki, I. Z., Frankel L. K., and Bricker, T. M. (1998) *Biochemistry 37*, 13553−13558.
44. Weinreb, P. H., Weiguo, X., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996) *Biochemistry 35*, 13709−13715.

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