Site-Directed Mutagenesis of Conserved C-Terminal Tyrosine and Tryptophan Residues of PsbO, the Photosystem II Manganese-Stabilizing Protein, Alters Its Activity and Fluorescence Properties[†]

Aaron J. Wyman,[‡,§] Hana Popelkova,[‡] and Charles F. Yocum*[,‡,||]

Department of Molecular, Cellular and Developmental Biology and Department of Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-1048

Received February 7, 2008; Revised Manuscript Received May 2, 2008

ABSTRACT: The extrinsic photosystem II PsbO subunit (manganese-stabilizing protein) contains near-UV CD signals from its complement of aromatic amino acid residues (one Trp, eight Tyr, and 13 Phe residues). Acidification, *N*-bromosuccinimide modification of Trp, reduction or elimination of a disulfide bond, or deletion of C-terminal amino acids abolishes these signals. Site-directed mutations that substitute Phe for Trp241 and Tyr242, near the C-terminus of PsbO, were used to examine the contribution of these residues to the activity and spectral properties of the protein. Although this substitution is, in theory, conservative, neither mutant binds efficiently to PSII, even though these proteins appear to retain wild-type solution structures. Removal of six residues from the N-terminus of the W241F mutant restores activity to near-wild-type levels. The near-UV CD spectra of the mutants are modified; well-defined Tyr and Trp peaks are lost. Characterizations of the fluorescence spectra of the full-length WF and YF mutants indicate that Y242 contributes significantly to PsbO's Tyr fluorescence emission and that an excited-state tyrosinate could be present in PsbO. Deletion of W241 shows that this residue is a major contributor to PsbO's fluorescence emission. Loss of function is consistent with the proposal that a native C-terminal domain is required for PsbO binding and activity, and restoration of activity by deletion of N-terminal amino acids may provide some insights into the evolution of this important photosynthetic protein.

Photosystem II (PSII)[1] contains intrinsic and extrinsic proteins and an inorganic ion cluster (four Mn, one Ca^{2+}, and one Cl^-) that is the site of H_2O oxidation (*1*). The largest extrinsic subunit, called PsbO or manganese-stabilizing protein, is found in prokaryotes and eukaryotes. It contains 247 amino acids, binds the other eukaryotic extrinsic subunits (PsbP and PsbQ), and stabilizes the Mn cluster (*2*). The PsbO molecular mass is 26.5 kDa; it contains one conserved S−S bond (*3, 4*) and behaves in solution as a natively unfolded or intrinsically disordered protein (*5, 6*). Proteins in this family possess unusual physical and chemical characteristics [thermostability, high ratios of charged to hydrophobic residues, secondary structures that are predicted to contain large amounts of random coil and turns, and extended solution structures (*7*)]. They also exhibit anomalous mobility in SDS−PAGE, abnormally large Stokes radii (R_s) and sedimentation coefficients, and very acidic or basic pI values (*7*). Despite similar structural and functional properties, the amino acid sequences of these proteins have no obvious similarities.

In PsbO, the intensities of near-UV CD signals from Trp, Tyr, and Phe, which depend on the hydrophobicity of their local environments (*8*), have proven to be useful probes of changes in PsbO solution structure (*4, 5, 9, 10*). Well-defined near-UV CD peaks at 291−293 and 285−287 nm in PsbO arise from Trp and Tyr residues in hydrophobic environments, and smaller peaks at 258−264 nm are assigned to Phe (*9, 11*). The 293 nm shoulder in the wild-type (WT) PsbO UV spectrum (*9, 12*) is also characteristic of Trp in a hydrophobic environment (*8, 13*). The lone Trp (W241) in spinach PsbO is conserved in other eukaryotic PsbO's and is replaced conservatively with Phe in prokaryotes (*3, 4*). The eight Tyr and 13 Phe residues in eukaryotic PsbO's are distributed throughout the amino acid sequence, but Y242 is located next to W241 in the primary sequence of the protein. Modification of W241 by *N*-bromosuccinimide (NBS) affects UV absorbance, far-UV CD, and fluorescence emission spectra, and the modified PsbO cannot bind effectively to PSII (*14, 15*). Phenylalanine replaces Trp in

[†] This research was supported by grants to H.P. and C.F.Y. from the National Science Foundation (MCB-0110455 and -0716541).

* To whom correspondence should be addressed: Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI 48109-1048. Telephone: (734) 647-0887. Fax: (734) 647-0884. E-mail: cyocum@umich.edu.

[‡] Department of Molecular, Cellular and Developmental Biology.

[§] Current address: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907.

[||] Department of Chemistry.

[1] Abbreviations: Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CD, circular dichroism; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IPTG, isopropyl β-D-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; NBS, *N*-bromosuccinimide; PsbO, manganese-stabilizing protein; OEC, oxygen-evolving complex; PSII, photosystem II; SW-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic proteins; Tris, tris(hydroxymethyl)aminomethane; USW-PSII, urea NaCl-washed photosystem II membranes depleted of PsbO, PsbP, and PsbQ (33, 23, and 17 kDa, respectively) extrinsic proteins; @, stop codon replacing the E246 codon in PsbO.

10.1021/bi800225m CCC: $40.75
Published on Web 05/24/2008

Thermosynechococcus elongatus PsbO, and its near-UV CD spectrum exhibits peaks between 260 and 290 nm from Tyr and Phe, including 274 and 282 nm peaks not seen in WT spinach PsbO (*4*). The pH, heat, and denaturation sensitivities of Trp and Tyr fluorescence emission from PsbO (*16–19*) suggest that the overall emission is comprised of signals from both C-terminal amino acids in hydrophobic environments, that the S−S bond acts as a quencher of Trp fluorescence, and that at high (11.3) pH, Y242 exists as a tyrosinate ion that also acts as a quencher (*16, 17*).

Deletion of C-terminal residues (Q244 and L245) weakened or eliminated PsbO binding to spinach PSII and eliminated near-UV CD peaks from Trp and Tyr (*9, 20*). Acidification of PsbO or reduction of the S−S bond (*11, 19, 21*) produces similar effects, and the near-UV CD spectrum of a PsbO mutant lacking the S−S bond (C28A/C51A PsbO) has no Trp or Tyr peaks (*10*); NBS modification of Trp in spinach PsbO (*14*) gives a similar response. Mutations at the S−S bond near the PsbO N-terminus and at the C-terminus yield similar solution conformations, suggesting that these domains may interact in solution, in agreement with other results (*6, 12, 22-24*).

Loss of near-UV CD signals upon mutation or chemical modification of PsbO raises the question of which of the eight Tyr residues in spinach PsbO is responsible for the signal in the near-UV CD spectrum of WT PsbO (*6, 23*). Because Y242 is highly conserved in all PsbO's, it has been proposed that this residue may produce the majority of the near-UV Tyr CD signal (*4, 11, 23*). To further characterize the properties of the Tyr and Trp near-UV CD signals in spinach PsbO and to probe the role of the C-terminus of PsbO in the protein's activity in greater detail, we introduced two mutations, W241F and Y242F. The activity and solution structures of these mutants were characterized, and fluorescence measurements were carried out. Both mutants exhibit weakened binding to PSII and reconstitute low O_2 evolution activities, although they possess near-WT levels of estimated secondary structure and similar apparent molecular masses. Deletion of six N-terminal amino acids from the W241F mutant restores activity.

MATERIALS AND METHODS

Mutations of psbO cDNA and Transformations of Escherichia coli Cells. Preparations of recombinant *psbO* cDNA sequences carrying mutations resulting in the replacement of W241 and Y242 with Phe, and their subsequent transformation into *E. coli* overexpression lines, were created using the procedures detailed by Popelkova et al. (*12*). For the recombinant W241F PsbO and Y242F PsbO mutants, the N-terminal oligonucleotide 5′-TCATATGGCAGCTTCATTACAAGCATC-3′ and the C-terminal oligonucleotides 5′-TTTTATTGCTCAAGTTGTGCATAAAAAAC-3′ and 5′-TTTTATTGCTCAAGTTGTGCAAACCA-3′ were synthesized (Gibco BRL or Invitrogen custom primers) on the basis of the sense and antisense strands of the PsbO N-terminal and C-terminal coding regions, respectively. After PCR amplification and cloning, the altered *psbO* cDNA sequences were confirmed by DNA sequencing. Edman degradation of the overexpressed mutant proteins yielded the expected N-terminal sequence [EGGK (data not shown)]. The N-terminal sequence of the truncated W241F mutant (ΔL6MW241F) was MTYDE, and the protein's mass, as determined by MALDI-TOF mass spectrometry, was 25984 ± 20 Da.

Overexpression and Purification of Recombinant W241F PsbO and Y242F PsbO. The W241F and Y242F mutants were overexpressed in *E. coli*, and the resulting inclusion bodies were isolated as described previously (*25, 26*), with the following modifications. The LB medium contained only ampicillin (50 μg/mL), and protein overexpression was induced using 25−32.5 μM IPTG. Cells expressing W241F PsbO and Y242F PsbO produced reduced levels of mutant PsbO in comparison to that of recombinant WT PsbO (data not shown); this has been reported for overexpression of other recombinant mutant proteins in which Trp and/or Tyr residues were replaced with Phe (*27*).

Inclusion bodies were solubilized with 3 M urea, 20 mM Bis-Tris (pH 6.4), and 5 mM NaCl (solubilization buffer) (*23, 25, 26*). The resulting suspension was centrifuged at 48000*g* for 30 min at 4 °C, and the PsbO-enriched supernatant was loaded onto a Pharmacia Resource Q column equilibrated with solubilization buffer containing 5% betaine. Application of a linear salt gradient (5 to 250 mM NaCl) eluted PsbO between 100 and 200 mM NaCl. Fractions containing PsbO were combined, dialyzed overnight against solubilization buffer without betaine, and loaded onto the same Resource Q column equilibrated with solubilization buffer containing 5% betaine; application of a step NaCl gradient (30, 70, 170, and 1000 mM NaCl) eluted PsbO at 170 mM NaCl. To prevent the aggregation of PsbO when it was frozen (−70 °C), the purified proteins were kept in solubilization buffer or were dialyzed overnight at 4 °C against a buffer containing 0.4 M sucrose, 50 mM MES (pH 6.0), and 10 mM NaCl (SMN buffer). The extinction coefficients of W241F PsbO (7.9 mM^{-1}) and Y242F PsbO (15.1 mM^{-1}) at 280 nm were determined by the method of Gill and von Hippel (*28*); these values were used for spectrophotometric assays of protein concentration. The W241F mutant, truncated by six amino acids (ΔL6MW241F PsbO), was obtained in the same way as full-length W241F except that overexpression was induced with 0.5 mM IPTG, and this mutant was solubilized and purified by FPLC anion exchange only once using a Tris buffer system [3 M urea, 20 mM Tris (pH 8), 5 mM NaCl, and 5% betaine]. SDS−PAGE showed that the WT and mutant proteins were >90% pure.

Reconstitution of PSII with Mutant PsbO's. Photosystem II membranes, SW-PSII, and USW-PSII were prepared as described by Popelkova et al. (*12*). The O_2 evolution rates were as follows: 600−700 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for PSII membranes, 300−400 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for SW-PSII, and 80−150 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} for USW-PSII. Reconstitution reactions and O_2 evolution assays using W241F PsbO, Y242F PsbO, and ΔL6MW241F PsbO were conducted as previously described (*20, 26, 29*) with the following modifications: 300 μM DCBQ was the acceptor in assays of control O_2 evolution rates of SW-PSII samples, while 600 μM DCBQ was used for assays of reconstituted samples. W241F and Y242F PsbO samples stored in solubilization buffer at −70 °C were dialyzed overnight against SMN buffer prior to use in reconstitution reactions; ΔL6MW241F was stored directly in SMN. The O_2 evolution rate assayed under saturating light conditions for 1 min monitored PSII turnover, while the O_2 evolution

yield was assayed at 80% light saturation for 4 min to determine the long-term stability of the reconstituted system. Analysis of mutant PsbO rebinding to PSII was carried out as previously described (*23, 26, 29*). The O_2 evolution activity and PsbO binding data are the average of three assays for each point on the graphs.

Circular Dichroism, UV Absorbance Spectroscopy, and Size-Exclusion Chromatography. The far-UV CD and near-UV CD spectra of W241F PsbO, Y242F PsbO, and ΔL6MW241F PsbO were measured and analyzed as described by Popelkova et al. (*12*) and in the legend of Figure 2. UV absorbance spectra were obtained using an OLIS-modified Cary-17 instrument, and the experimental conditions are given in the legend of Figure 3. Size-exclusion chromatography of W241F and Y242F PsbO was carried out as described by Popelkova et al. (*12*), except that the column was equilibrated with 20 mM Tris (pH 8.0) and 50 mM NaCl to prevent PsbO from aggregating (*26*).

Fluorescence Spectroscopy. W241F and Y242F PsbO were dialyzed against 10 mM KH_2PO_4 (pH 6.0) for analysis of their fluorescence excitation and emission spectra using a Hitachi FL-4500 fluorescence spectrophotometer. The software supplied by the manufacturer was used to record and analyze the data. A 1.5 mL quartz cuvette with a path length of 1.0 cm was used; each spectrum represents the average of three replicate scans from which the buffer signals were subtracted. Additional conditions are given in the figure legends.

RESULTS

Reconstitution of O_2 Evolution Activity by W241F and Y242F PsbO. Figure 1 shows that W241F restored O_2 rates and yields to 5−10% of SW-PSII control activities at a level of 2 mol of W241F PsbO/mol of PSII (the WT/PSII ratio), increasing to 25% of the SW control after incubation with 8 mol of PsbO/mol of PSII; binding failed to saturate. Incubation of PsbO-depleted PSII with 8 mol of W241F PsbO/mol of PSII resulted in binding of only 1 mol of protein, which indicates a significant assembly defect. The possibility that W241F was cold-sensitive, like V235A and E246@ PsbO mutants (*20, 29*), was examined; no enhancement of reconstitution of PSII activity was observed at 22 °C (data not shown). Experiments with Y242F yielded nearly identical results (Figure 1); 2 mol of Y242F PsbO/mol of PSII reconstituted 5−10% of control activity, which increased to 30% at 8 mol of PsbO/mol f PSII. Approximately 1.3−1.4 mol of Y242F bound after incubation with 8 mol of protein/mol of PSII; binding was temperature-insensitive (data not shown). These results resemble those reported for NBS-modified PsbO (*14*) and for PsbO C-terminal deletion mutants (*6, 9, 20*). The W241F mutation creates a partial sequence that is homologous to the C-terminal PsbO sequences found in cyanobacteria (*4*). Cyanobacterial PsbO is truncated at the N-terminus relative to eukaryotic PsbO's (*6*), so a new mutation (ΔL6MW241F) was created to remove part of the N-terminus. As Figure 1 shows, this mutation binds to PSII and reconstitutes activity at 22 °C to nearly the same levels as WT PsbO. The mutant is partially cold-sensitive; activity reconstitution is 20% lower at 4 °C.

Solution Structure of W241F and Y242F PsbO's. Far-UV CD spectra of W241F and Y242F PsbO are very similar to

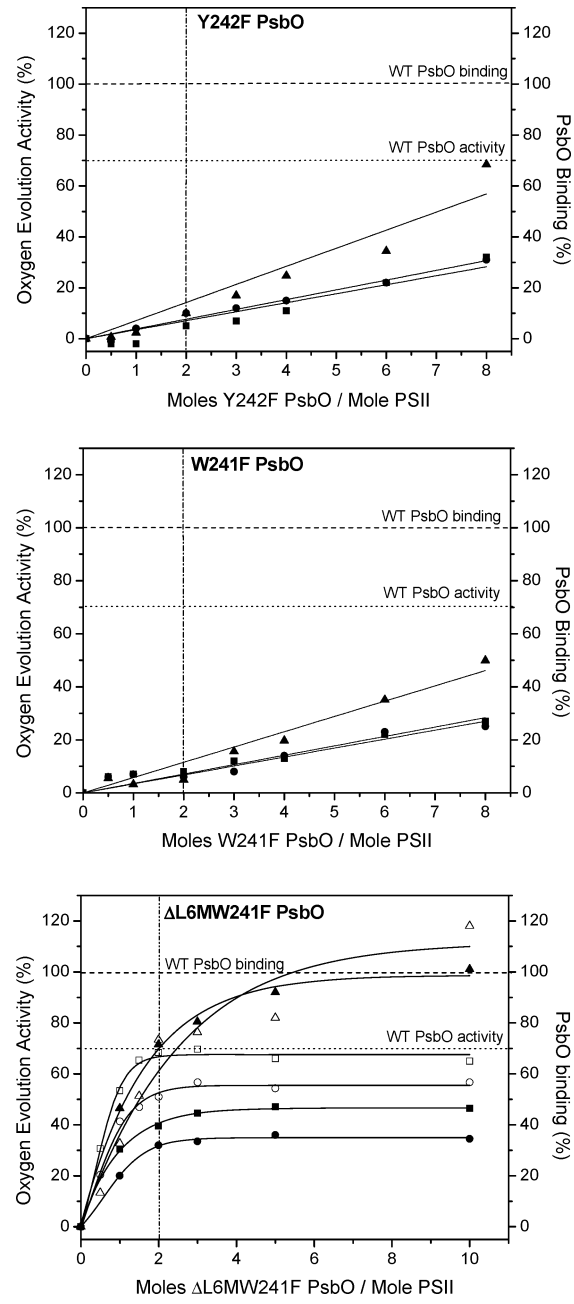

FIGURE 1: Reconstitution of USW-PSII preparations with recombinant Y242F PsbO, W241F PsbO, and ΔL6MW241F PsbO. PsbO binding curves are shown (▲). The O_2 rates (●) and O_2 yields (■) were obtained from assays carried out under continuous illumination for 1 and 4 min, respectively. After reconstitution for 1 h on ice (filled symbols) or 22 °C (empty symbols), samples were assayed for activity; the residual activity of USW-PSII was subtracted from that of the reconstituted samples. The control (100% activity) is that of a SW-PSII sample. Dotted lines indicate WT PsbO activity, while dashed lines show WT PsbO binding. All WT levels normally saturate at 2 mol of WT PsbO/mol of PSII (*23, 26*).

those of WT PsbO (data not shown); Table 1 presents data on the thermostability of the estimated secondary structure contents of both mutants. At 90 °C, the CD data predict that the proteins gain small amounts of random coil and α-helix at the expense of β-sheet and regain most of their original secondary structures upon cooling. However, Table 1 also shows that substitution of Phe for either Tyr or Trp stabilizes the β-sheet content; heating causes somewhat smaller losses of this secondary structure element than we observed with WT PsbO. Near-UV CD spectra (Figure 2) of W241F PsbO

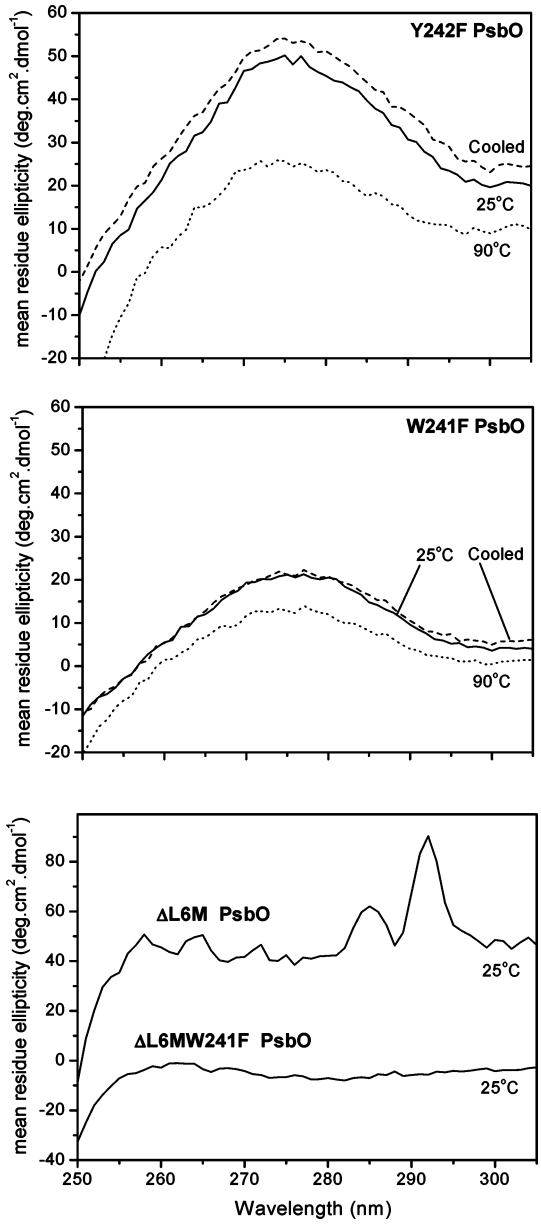

FIGURE 2: Effect of heating on the near-UV CD spectra of W241F PsbO and Y242F PsbO. Spectra were recorded after allowing the proteins to equilibrate to the indicated temperature. Each spectrum is the average of 20 scans. The protein concentrations were 51.2 μM for W241F and 26.8 μM for Y242F. The buffer in both experiments was 10 mM KH_2PO_4; the pH was 6.0 or 5.7, as determined from the buffer's temperature coefficient at 25 and 90 °C. Each spectrum was recorded at 25 and 90 °C and after cooling to 25 °C. The bottom panel presents the near-UV CD spectra of ΔL6MW241F PsbO (40 μM) and ΔL6M PsbO (10 μM) in 10 mM KH_2PO_4 at pH 6.0 and 25 °C. The ΔL6M spectrum is offset by 75 units positive for clarity of presentation. Experimental conditions for all spectra were as follows: scan width, 320−250 nm; time constant, 1 s; bandwidth, 1 nm; path length, 1 cm; sample volume, 1 mL.

and Y242F PsbO lack the well-defined Trp and Tyr peaks at 291−293 and 285−287 nm, respectively, and the weak Phe peaks centered at 258 and 264 nm in WT PsbO (*4–6, 11, 12, 19, 23*). The broad peak centered at 275−276 nm in both mutant proteins is unusual; if W241 and Y242 reside in a hydrophobic environment, one might expect replacement of either residue with Phe to produce a more intense peak at 258−266 nm. Heating both mutants to 90 °C depresses the signals' intensities, but in contrast to loss of the Trp and

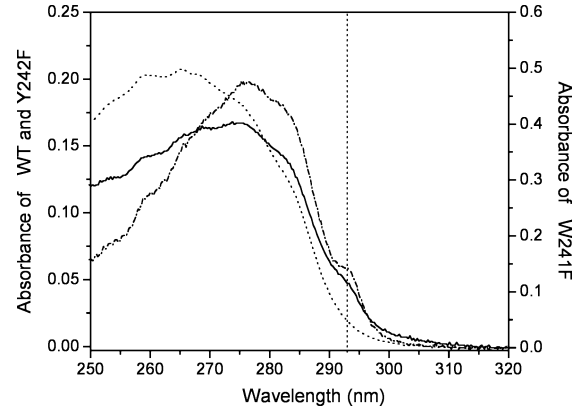

FIGURE 3: UV absorption spectra of WT PsbO, W241F PsbO, and Y242F PsbO. The protein concentrations were 12.9 μM for WT, 51.8 μM for W241F, and 12.5 μM for Y242F; a higher concentration of W241F was used to emphasize the absence of the shoulder at 293 nm. The proteins were dissolved in 10 mM KH_2PO_4 buffer (pH 6.0). The spectra are those for (-•-) WT PsbO, (—) Y242F PsbO, and (•••) W241F PsbO. The vertical dotted line indicates the 293 nm position of the Trp shoulder.

Table 1: Secondary Structure Predictions for WT PsbO, W241F PsbO, and Y242F PsbO Based on Analyses of Far-UV CD Spectra[a]

protein	α-helix (%)	β-sheet (%)	turn + unordered (%)	total (%)
WT (25 °C)	4	36	60	100[b]
WT (90 °C)	6	25	68	99[b]
WT (cooled)	3	37	59	99[b]
W241F (25 °C)	3	38	58	99
W241F (90 °C)	3	37	59	99
W241F (cooled)	2	42	53	97
Y242F (25 °C)	3	39	57	99
Y242F (90 °C)	4	33	62	99
Y242F (cooled)	3	37	59	99

[a] The numbers are averages of results obtained from the basis sets shown and from both CONTIN/LL and CDSSTR methods. Basis sets 6 and 7, which contain secondary structure contents from five denatured proteins, were used to estimate secondary structure. [b] Data from ref *10*.

Tyr peaks at 90 °C in WT PsbO (*5, 6*), neither signal in the mutant proteins disappears completely. The near-UV CD spectra of both mutants are restored upon cooling (Figure 2). Figure 2 also presents the near-UV CD spectra for ΔL6MW241 and ΔL6M PsbO, the control for this particular mutant that exhibits spectral peaks even more pronounced than those of WT (*23*). The W241F substitution eliminates the near-UV CD signal from tyrosine(s). The fact that the ΔL6MW241F mutant is highly active would suggest that Tyr residues other than Y242 are unaffected by this mutation and that Y242 makes the dominant contribution to the near-UV CD spectrum of PsbO.

Size-exclusion chromatography and SDS−PAGE indicate that both full-length mutants have electrophoretic mobilities and molecular masses similar to that of WT PsbO (*5, 6, 9, 12, 20, 23*) (data not shown). The solution structures of W241F PsbO and Y242F PsbO were also analyzed by UV absorption spectroscopy (Figure 3). The UV absorbance spectrum of Y242F PsbO bears some resemblance to the WT spectrum; however, the Trp shoulder at 293 nm and Tyr absorbance exhibit decreased intensities, and Phe absorbance has increased, resulting in a blue-shifted peak at 274 nm. The two peaks assigned to Phe remain at 259 and 266 nm, and increased UV absorbance is present at lower wavelengths. This is probably due to the Y → F substitution;

nuclease treatments were used to minimize DNA contamination, and SDS−PAGE and far-UV CD spectra failed to detect protein or nucleic acid contaminations in either mutant protein. The spectrum of W241F is also substantially altered; the 293 nm shoulder is missing, and Phe absorbance produces a broad peak at 266 nm. The spectrum of this mutant, taken at a higher protein concentration (51.8 μM), emphasizes the absence of absorbance features at 290−300 nm, which eliminates the possibility of contamination by other Trp-containing proteins. Like Y242F, the W241F spectrum exhibits stronger absorption at 250 nm than the WT protein, and the absorption peaks due to Phe are enhanced. Taken together, these data indicate that the overall tertiary structures of W241F and Y242F and their thermostabilities are similar to those of WT PsbO. The mutants retain some near-UV CD signals at high temperatures, and this and the UV absorption spectra suggest that the local environment of W241 and Y242 may be altered when either residue is replaced with Phe. The UV absorption spectrum of the truncated W241F mutant (ΔL6MW241F) lacks the 293 nm shoulder (data not shown), as well as the near-UV CD signal from Tyr (Figure 2).

Properties of Fluorescence Excitation and Emission Associated with Y242F and W241F PsbO. The fluorescence properties of WT spinach PsbO contain contributions from Tyr and Trp (*11, 14–19, 21, 30, 31*). Figure S1 (Supporting Information) presents excitation spectra of WT, W241F, and Y242F PsbO collected at the emission wavelengths indicated by the labels on the curves. The mutant proteins exhibit decreased fluorescence intensities relative to that of WT PsbO. The strongest emission intensities (300−320 nm) in W241F arise from Tyr excitation at 277 nm (*13*). The excitation spectra for longer emission wavelengths have a maximum at 280−281 nm but yield weaker emissions. The Y242F mutant produced the strongest emission between 300 and 320 nm when excited at 277 nm and exhibited fluorescence emission of greater intensity at longer wavelengths (340−360 nm) than did W241F. The peak excitation (281−282 nm) for the longer wavelength emissions is expected for Trp excitation (*13*). No fluorescence emission from excitation of Phe was observed in either mutant (data not shown).

Overlapping absorbance spectra of Tyr and Trp, and the approximately 4-fold higher quantum yield of the Trp emission (*13*), make it difficult to determine the contributions of these residues to overall protein fluorescence emission. To further characterize the emission spectra of mutant and WT PsbO's, excitation at 275 nm was employed to elicit emission from Tyr and Trp (Figure 4A). The emission spectrum of WT PsbO, with a peak at 310 nm and a 310 nm/350 nm ratio of 3, matches spectra reported previously (*16, 17, 19*) for the protein isolated by either $CaCl_2$ or urea extraction procedures (*17, 19*); PsbO isolated by alkaline Tris washing has an emission peak that occurs at a slightly shorter wavelength, and a 310 nm/350 nm ratio of >3 (*30*). In contrast, the emission spectrum of Y242F PsbO exhibits a less intense peak at 312 nm, consistent with loss of the Tyr emission excited at this wavelength. Greater prominence of Trp emission, including a shoulder centered at 340 nm that is obscured in the WT emission spectrum, is present in this mutant. The emission spectrum of W241F PsbO is decreased in intensity in comparison to those of WT and Y242F PsbO

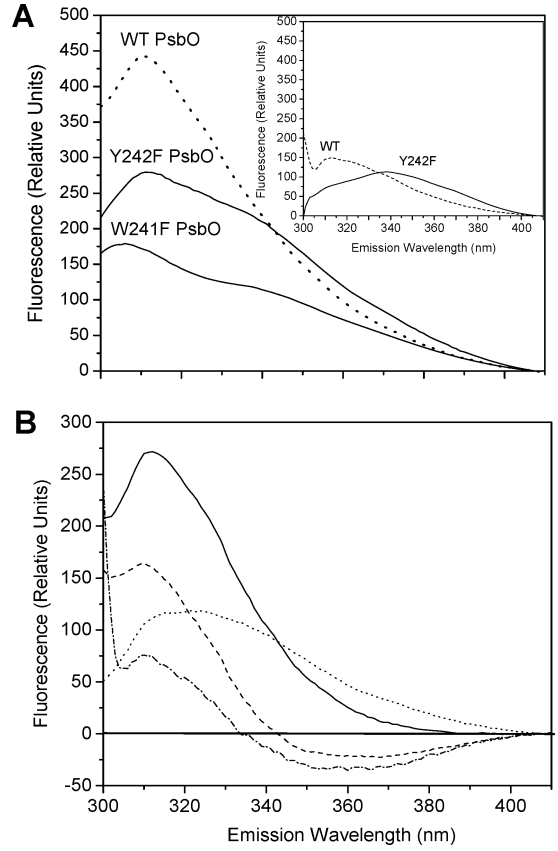

FIGURE 4: (A) Fluorescence emission spectra of WT PsbO, W241F PsbO, and Y242F PsbO (4.1 μM) in 10 mM KH_2PO_4 buffer (pH 6.0). Spectra were recorded at 25 °C with excitation at either 275 or 295 nm (inset). (Spectra in the inset from 300 to 305 nm include some interference from overlap between slits used for the excitation and emission monochromaters.) Experimental conditions: scan speed, 60 nm/min; delay, 1 s; excitation slit width, 5.0 nm; emission slit width, 5.0 nm; sample volume, 0.6 mL. (B) Difference emission spectra of WT PsbO, W241F PsbO, and Y242F PsbO (4.1 μM) in 10 mM KH_2PO_4 buffer (pH 6.0). Excitation was at 275 nm, and the spectra are shown for (•••) Y242F − W241F, (—) WT − W241F, and (---) WT − Y242F. Also shown is the emission spectrum of WT − Y242F (-•-) produced by excitation at 295 nm. All difference spectra are subtractions of the relative fluorescence emissions of equivalent concentrations (4.1 μM) of the various PsbO species.

(Figure 4A). A prominent blue-shifted peak at 307 nm from Tyr emission (*13*) is present, along with a small shoulder at 345 nm that would ordinarily be assigned to Trp emission. Emission spectra from WT and Y242F produced by selective Trp excitation at 295 nm (inset, Figure 4A) show that the broad WT emission is similar to that of the native protein in solution (*16, 17, 19, 30*), and that removal of Y242 causes the appearance of a broad peak centered at 338 nm in Y242F (*15, 16, 31*). The emission spectrum of W241F excited at 295 nm is not shown; proteins lacking Trp fail to produce significant fluorescence when excited at 295 nm (*27, 32*).

To evaluate the relative contributions of W241 and Y242, excited at 275 nm, to the emission spectrum of WT PsbO, difference spectra (WT − W241F and WT − Y242F) were produced (Figure 4B), using data from Figure 4A that were collected at identical instrument settings and protein concentrations. The WT − W241F spectrum indicates that W241 contributes a broad emission signal, from 310 to 390 nm, to the WT PsbO spectrum. The WT − Y242F spectrum has a

peak at 308 nm, as expected for Tyr, and the emission extends to 340 nm, after which a broad, negative feature centered at 360 nm appears, which is likely to originate from additional Trp fluorescence when Y242 is replaced with Phe. Signals from the seven remaining Tyr residues are subtracted from this spectrum, so their contribution to the WT PsbO spectrum cannot be determined. It is clear, however, that a significant fraction of the Tyr fluorescence emission from WT PsbO comes from Y242, because the WT − Y242F difference spectrum has a peak fluorescence intensity that is 35% of the control WT PsbO emission intensity at 308−310 nm (Figure 4B). The WT − Y242F difference spectrum produced by excitation at 295 nm (Figure 4B) removes most of the contribution of Y242 to the WT PsbO emission spectrum produced by excitation at this wavelength (Figure 4A) (*15, 16, 19, 21, 31*). The emission intensity is greatest at 308 nm and decreases rapidly at longer wavelengths; the appearance of a broad negative feature in the difference spectrum, centered at 360 nm, could represent additional emission from W241. Finally, the Y242F − W241F difference spectrum (excitation of each protein at 275 nm) allows one to estimate the fluorescence emission from W241 in the absence of Y242. This spectrum represents the subtraction of the fluorescence emission of eight Tyr residues from the emission of seven Tyr residues and W241, in the absence of Y242. The resulting spectrum has a peak at 322 nm and a broad shoulder that extends to 400 nm. In spite of the subtraction of the emission from Y242 in this spectrum, the result shown here clearly originates from Trp emission that is stimulated by removal of Tyr 242.

The effect of heating−cooling cycles (25 to 55 to 90 to 25 °C) on fluorescence emissions from W241F and Y242F PsbO, excited at 275 nm, is shown in Figure 5. When the compounds were heated, the emission spectrum of Y242F PsbO exhibited a loss of signal from Trp (312 nm) and a red shift (to 350 nm) of the signal intensity. Substantial emission intensity was regained after cooling. At 90 °C, the Tyr emission at 307 nm in W241F PsbO decreases, as does the magnitude of the shoulder at 340 nm. The majority of the Tyr signal is recovered upon cooling, but the emission spectrum from 325 to 360 nm is modified; the shoulder centered at 340 nm is lost (*33–35*). Heat-induced unfolding of W241F PsbO could alter the environment around Y242 so that upon refolding in solution, it is no longer in its native environment and fluorescence emission is thus decreased or lost (*33–35*). The absence of Trp eliminates this residue as a source of the 335−345 nm shoulder in the emission spectrum of W241F PsbO (Figure 4A).

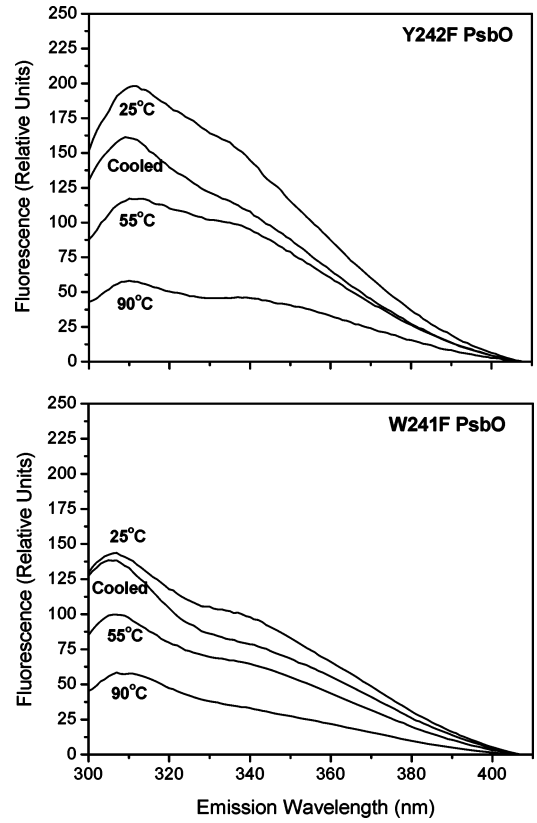

FIGURE 5: Effect of heating on the fluorescence emission spectra of W241F PsbO and Y242F PsbO. Changes in the fluorescence emission spectra of W241F (4.1 μM) and Y242F (4.1 μM) in 10 mM KH_2PO_4 buffer at pH 6.0 or 5.7, determined by the buffer's temperature coefficient, were monitored at 55 and 90 °C, and after cooling to 25 °C in a thermostated cuvette chamber. The proteins were excited at 275 nm. Experimental conditions: scan speed, 60 nm/min; delay, 1 s.

DISCUSSION

Spectroscopic Properties of the Aromatic Amino Acid Residues of PsbO. Near-UV CD spectra of W241F and Y242F PsbO lack the sharply defined features arising from Trp and Tyr in the WT protein (Figure 2). Native proteins and other W → F or Y → F mutants also exhibit near-UV CD spectra containing broad peaks between 274 and 276 nm (*4, 32, 36*) resembling those of W241F and Y242F PsbO (*37, 38*). These broad peaks, proposed to originate from interactions between Tyr and/or Trp and Phe, are affected by factors such as the distance between aromatic residues and the local environment (*4, 36, 38*). The similarity of the near-UV CD spectra of the mutants may indicate that W241, Y242, or the substituted F241 and F242 residues exist in the proximity of one another. The blue-shifted UV absorption spectrum of W241F PsbO (Figure 3) and the loss of the shoulder centered at 293 nm are consistent with the absence of Trp in a hydrophobic environment. The Y241F spectrum retains a peak at 276 nm, consistent with the presence of absorbance from several Tyr residues in PsbO. Both mutant proteins have prominent absorption bands in the 250−265 nm range arising from Phe and also exhibit an increased absorbance at 250 nm relative to their peak absorbance values. While the presence of a small amount of contaminants in these preparations cannot be excluded, their purity suggests that this absorbance may be a consequence of the Phe substitutions.

The fluorescence emission properties of W241F and Y242F (Figure 4A) indicate that both residues reside in a hydrophobic environment, in agreement with a number of other results (*9, 14–18, 23, 31*); W241 seems to be a major contributor to PsbO emission upon excitation at either 275 or 295 nm (inset), and Y242 (among the eight Tyr residues of PsbO) is likely to be a major source of PsbO's Tyr fluorescence emission (compare WT and Y242F emission spectra in Figure 4A). Heat-induced loss of fluorescence emission from both mutants (Figure 5) suggests that these residues reside in a hydrophobic domain. The Trp emission excited at 295 nm in Y242F (Figure 4A, inset) is better defined and intense that from WT PsbO, which could be

consistent with a loss of quenching of W241 fluorescence. This quenching could, in theory, be due to the disulfide bond; similar phenomena have been found in the DsbA protein, and in the enzyme cutinase (*32, 39*). Spatial neighbor analysis of 131 nonhomologous single-chain proteins showed that Trp is preferentially located near disulfide bonds (*40*). The 1.0 Å resolution crystal structure of *Fusarium solani* cutinase shows that the aromatic ring of the only Trp is located 5 Å from the S−S bond (*41*). The backbone three-dimensional structure of PsbO from *T. elongatus,* which superimposes well on the homologous three-dimensional model of spinach PsbO (*3*), predicts a similar distance between F239 (homologous to W241 in spinach) and the disulfide bond (*24*). If the S−S bond functions as a quencher of Trp fluorescence in WT PsbO, then release of quenching of W241 emission in Y242F PsbO could be caused by subtle structural changes induced by the Y → F mutation that shift W241 away from the disulfide bridge.

Although W241F, excited at 275 nm (Figure 4A), exhibits decreased fluorescence emission intensity, a broad featureless shoulder centered at 340 nm is present that cannot be attributed to Trp. It is possible that Y242 may produce an excited-state tyrosinate [$\epsilon = 2330\ M^{-1}\ cm^{-1}$ at 295 nm (*42*)] at the pH (6) of the buffer used in these experiments. This species is unlikely to have any effect on W241 fluorescence emission, unlike the situation for a ground-state tyrosine, which clearly acts as a fluorescence quencher in PsbO at pH 11 (*17*). In fact, the absence of a ground-state tyrosinate in W241F PsbO is consistent with its absorption spectrum, which lacks the absorbance (0.10, assuming an extinction coefficient of 2330 $M^{-1}\ cm^{-1}$) at 295 nm (Figure 3) that would be produced by a single tyrosinate ion (*42*).

Structural and Functional Consequences of Phe Mutations. Replacement of either W241 or Y242 of PsbO with Phe has little effect on the size of the recombinant proteins (data not shown) and produces minimal changes in the estimated secondary structure as monitored by far-UV CD (Table 1). Nevertheless, neither protein binds efficiently to PSII (Figure 1), giving only low levels of reconstituted O_2 evolution activity. These are among the first spinach PsbO mutants with native N-terminal sequences and WT solution structures (*6, 23*) that are impaired in their ability to bind to PSII and restore activity. The effects of mutating W241 and Y242 are consistent with results obtained with other C-terminal domain mutations of PsbO that affect activity (*6, 9, 14, 15, 20, 31*), and with the reports that W → F and/or Y → F mutations in other proteins create losses of function (*27*). In spite of the far-UV CD data showing that there has been little change in their secondary structure content, the near-UV CD spectra lack the well-defined Trp and Tyr peaks of the spectra of the WT protein (*5*).

The response to heating of both the near- and far-UV CD spectra of Y242F and W241F PsbO shows that substitution of Phe at either position yields a modest increase in the thermostability of the proteins' β-sheet content (Table 1), and in the stability of the C-terminal domain where these residues reside. These observations may provide insight into why these mutants fail to bind efficiently to PSII. Wild-type PsbO gains β-sheet structure upon binding to PSII (*43*), and it is possible that the additional stability of these mutants interferes with the refolding events required for productive binding to PSII. Mutations that substitute Phe for Trp cause an assortment of effects on protein stability, from none to adverse (*44, 45*), or small increases (*46*). Similar effects have been attributed to Y → F mutations (*47*).

Deletion of a short N-terminal sequence of PsbO which is part of a domain that is predicted to interact with the C-terminus of spinach PsbO (*6, 22*) to produce the ΔL6MW241F mutation produces multiple effects on the W241F mutation. Figures 1 and 2 show that WT binding and activity reconstitution are restored, and that the Tyr near-UV CD signal is lost, indicating a perturbation to the C-terminal domain of the protein. This must be due to the combined replacement of Trp with Phe and loss of a short N-terminal segment of the protein which, in the wild type, has little effect on activity or binding of PsbO (*23*). These results are consistent with a model for PsbO structure in which a certain amount of flexibility is required for productive binding and refolding of the protein into its PSII-associated tertiary structure. They are also in agreement with the model from the crystal structure of PSII (*3*) that predicts that the N- and C-termini are close to one another, although the solution structure of PsbO would be less compact (*5*) than the PSII-bound form. The results with ΔL6MW242F suggest that introduction of Phe at position 241 may restrict this flexibility, which is restored by deletion of six N-terminal amino acids from the protein. It is possible that these results also reflect an evolutionary adaptation to eukaryotic PsbO that contains an additional N-terminal sequence that is required for binding to PSII, a sequence that is absent from the cyanobacterial proteins (*6, 12*). It is possible that addition of this sequence during evolution also required the substitution of Trp for Phe in the eukaryotic sequence to maintain solution structure flexibility for productive assembly of PsbO. ΔL6MW242F also exhibits a moderate (20% inhibition of reconstitution) sensitivity to low temperature; this is less dramatic than the effect seen with the V235A mutation (*29*) but demonstrates again the importance of the C-terminal domain of PsbO in the functional assembly of the protein into PSII.

The data presented here provide additional evidence of the presence of a hydrophobic domain in PsbO that includes W241 and Y242. These residues confer a minimal stability on the protein's solution structure but also appear to allow for the rearrangements in secondary and tertiary structure needed for high-affinity binding of PsbO to its contact sites in PSII. The ability of W → F or Y → F mutations to interfere with PsbO binding and the reversal of this inhibition by a second-site truncation mutation (ΔL6M) in W241F suggest that interactions between the C- and N-termini of the protein are critical factors not only for the maintenance of solution structures but also in allowing the refolding of PsbO when it forms its high-affinity interactions with PSII. Our data suggest that in cyanobacteria, PsbO sequences at the C-terminus may contain a Phe residue in place of Trp at the homologous position in the primary sequence to stabilize the protein against stress (for example, elevated temperatures in thermophiles), and as a consequence, a truncated N-terminus was favored in these organisms to permit facile binding to PSII. In eukaryotes, Trp replaces Phe and an extended N-terminus was permitted under these conditions because Trp at position 241 allows greater flexibility in refolding of the protein during binding and assembly into PSII.

ACKNOWLEDGMENT

We thank Prof. Ursula Jakob for the generous use of the fluorimeter and related equipment and Paul Graf for assistance in the measurements.

SUPPORTING INFORMATION AVAILABLE

Fluorescence excitation spectra of WT, W241F, and Y242F PsbO's. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Nelson, N., and Yocum, C. F. (2006) Structure and function of photosystems I and II. *Annu. Rev. Plant Biol. 57*, 521–565.
2. Bricker, T. M., and Frankel, L. K. (1998) The structure and function of the 33 kDa extrinsic protein of photosystem II: A critical assessment. *Photosynth. Res. 56*, 157–173.
3. De Las Rivas, J., and Barber, J. (2004) Analysis of the structure of the PsbO protein and its implications. *Photosynth. Res. 81*, 329–343.
4. Motoki, A., Usui, M., Shimazu, T., Hirano, M., and Katoh, S. (2002) A domain of the manganese-stabilizing protein from *Synechococcus elongatus* involved in functional binding to photosystem II. *J. Biol. Chem. 277*, 14747–14756.
5. Lydakis-Simantiris, N., Hutchison, R. S., Betts, S. D., Barry, B. A., and Yocum, C. F. (1999) Manganese stabilizing protein of photosystem II is a thermostable, natively unfolded polypeptide. *Biochemistry 38*, 404–414.
6. Popelkova, H., Wyman, A., and Yocum, C. (2003) Amino acid sequences and solution structures of manganese stabilizing protein that affect reconstitution of photosystem II activity. *Photosynth. Res. 77*, 21–34.
7. Uversky, V. N. (2002) What does it mean to be natively unfolded? *Eur. J. Biochem. 269*, 2–12.
8. Kelly, S. M., and Price, N. C. (1997) The application of circular dichroism to studies of protein folding and unfolding. *Biochim. Biophys. Acta 1338*, 161–185.
9. Lydakis-Simantiris, N., Betts, S. D., and Yocum, C. F. (1999) Leucine 245 is a critical residue for folding and function of the manganese stabilizing protein of photosystem II. *Biochemistry 38*, 15528–15535.
10. Wyman, A. J., and Yocum, C. F. (2005) Structure and activity of photosystem II manganese-stabilizing protein: Role of the conserved disulfide bridge. *Photosynth. Res. 85*, 359–372.
11. Shutova, T., Irrgang, K.-D., Shubin, V., Klimov, V. V., and Renger, G. (1997) Analysis of pH-induced structural changes of the isolated extrinsic 33 kilodalton protein of photosystem II. *Biochemistry 36*, 6350–6358.
12. Popelkova, H., Im, M. M., D'Auria, J., Betts, S. D., Lydakis-Simantiris, N., and Yocum, C. F. (2002) N-Terminus of the photosystem II manganese stabilizing protein: Effects of sequence elongation and truncation. *Biochemistry 41*, 2702–2711.
13. Schmidt, F. X. (1999) Optical Spectroscopy to Characterize Protein Conformation and Conformational Changes. In *Protein Structure: A Practical Approach* (Creighton, T. E., Ed.) pp 261−297, IRL Press, New York.
14. Yu, Y., Li, R., Xu, Ch., Ruan, K., Shen, Y., and Govindjee (2001) N-Bromomsuccinimide modification of tryptophan 241 at the C-terminus of the manganese stabilizing protein of plant photosystem II influences its structure and function. *Physiol. Plant. 111*, 108–115.
15. Weng, J., Tan, C., Shen, J.-R., Yu, Y., Zeng, X., Xu, C., and Ruan, K. (2004) pH-induced conformational changes in the soluble manganese-stabilizing protein of photosystem II. *Biochemistry 43*, 4855–4861.
16. Shutova, T., Deikus, G., Irrgang, K.-D., Klimov, V. V., and Renger, G. (2001) Origin and properties of fluorescence emission from the extrinsic 33 kDa manganese stabilizing protein of higher plant water oxidizing complex. *Biochim. Biophys. Acta 1504*, 371–378.
17. Shutova, T., Villarejo, A., Zietz, B., Klimov, V. V., Gillbro, T., Samuelsson, G., and Renger, G. (2003) Comparative studies on the properties of the extrinsic manganese-stabilizing protein from higher plants and of a synthetic peptide of its C-terminus. *Biochim. Biophys. Acta 1604*, 95–104.
18. Shutova, T., Nikitina, J., Deikus, G., Andersson, B., Klimov, V. V., and Samuelsson, G. (2005) Structural dynamics of the manganese-stabilizing protein: Effect of pH, calcium, and manganese. *Biochemistry 44*, 15182–15192.
19. Tanaka, S., Kawata, Y., Wada, K., and Hamaguchi, K. (1989) Extrinsic 33-kdalton protein of spinach oxygen-evolving complexes: Kinetic studies of folding and disulfide reduction. *Biochemistry 28*, 7188–7193.
20. Betts, S. D., Lydakis-Simantiris, N., Ross, J. R., and Yocum, C. F. (1998) 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. *Biochemistry 37*, 14230–14236.
21. Zhang, L. X., Liang, H. G., Wang, J., Li, W. R., and Yu, T. Z. (1996) Fluorescence and Fourier-transform infrared spectroscopic studies on the role of disulfide bond in the calcium binding in the 33 kDa protein of photosystem II. *Photosynth. Res. 48*, 379–384.
22. 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) Intramolecular cross-linking of the extrinsic 33-kDa protein leads to loss of oxygen evolution but not its ability of binding to photosystem II and stabilization of the manganese cluster. *J. Biol. Chem. 273*, 4629–4634.
23. Popelkova, H., Im, M. M., and Yocum, C. F. (2003) Binding of manganese stabilizing protein to photosystem II: Identification of essential N-terminal threonine residues and domains that prevent nonspecific binding. *Biochemistry 42*, 6193–6200.
24. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature 438*, 1040–1044.
25. Betts, S. D., Hachigian, T. M., Pichersky, E., and Yocum, C. F. (1994) Reconstitution of the spinach oxygen-evolving complex with recombinant *Arabiodopsis* manganese-stabilizing protein. *Plant Mol. Biol. 26*, 117–130.
26. Betts, S. D., Ross, J. R., Hall, K. U., Pichersky, E., and Yocum, C. F. (1996) Functional reconstitution of photosystem II with recombinant manganese-stabilizing proteins containing mutations that remove the disulfide bridge. *Biochim. Biophys. Acta 1274*, 135–142.
27. de Antonio, C., del Pozo, A. M., Mancheno, J. M., Onaderra, M., Lacadena, J., Martinez-Ruiz, A., Perez-Canadillas, J. M., Bruix, M., and Gavilanes, J. G. (2000) Assignment of the contribution of the tryptophan residues to the spectroscopic and functional properties of the ribotoxin α-sarcin. *Proteins: Struct., Funct., Genet. 41*, 350–361.
28. Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino-acid sequence data. *Anal. Biochem. 182*, 319–326.
29. Betts, S. D., Ross, J. R., Pichersky, E., and Yocum, C. F. (1996) Cold-sensitive assembly of a mutant manganese-stabilizing protein caused by a Val to Ala replacement. *Biochemistry 35*, 6302–6307.
30. Kruk, J., Burda, K., Jemiola-Rzeminska, M., and Strzalka, K. (2003) The 33 kDa protein of photosystem II is a low-affinity calcium- and lanthanide-binding protein. *Biochemistry 42*, 14862–14867.
31. Zhang, F., Gao, J., Weng, J., Tan, C., Ruan, K., Xu, C., and Jiang, D. (2005) Structural and functional differentiation of three groups of tyrosine residues by acetylation of *N*-acetylimidazole in manganese stabilizing protein. *Biochemistry 44*, 719–725.
32. Hennecke, J., Sillen, A., HuberWunderlich, M., Engelborghs, Y., and Glockshuber, R. (1997) Quenching of tryptophan fluorescence by the active-site disulfide bridge in the DsbA protein from *Escherichia coli*. *Biochemistry 36*, 6391–6400.
33. Szabo, A. G., Lynn, K. R., Krajcarski, D. T., and Rayner, D. M. (1978) Tyrosinate fluorescence maxima at 345 nm in proteins lacking tryptophan at pH 7. *FEBS Lett. 94*, 249–252.
34. Prendergast, F. G., Hampton, P. D., and Jones, B. (1984) Characteristics of tyrosinate fluorescence emission in α-purothionins and *β*-purothionins. *Biochemistry 23*, 6690–6697.
35. Wilke-Mounts, S., Weber, J., Grell, E., and Senior, A. E. (1994) Tryptophan-free *Escherichia-Coli* F1-ATPase. *Arch. Biochem. Biophys. 309*, 363–368.
36. Searcy, D. G., Montenaygarestier, T., Laston, D. J., and Helene, C. (1988) Tyrosine environment and phosphate binding in the archaebacterial histone-like protein HTa. *Biochim. Biophys. Acta 953*, 321–333.
37. Welfle, H., Misselwitz, R., Welfle, K., Schindelin, H., Scholtz, A. S., and Heinemann, U. (1993) Conformations and conformational-changes of 4 Phe→Trp variants of the DNA-binding histone-

like protein, Hbsu, from *Bacillus subtilis* studied by circular dichroism and fluorescence spectroscopy. *Eur. J. Biochem. 217*, 849–856.
38. Koepf, E. K., Petrassi, H. M., Sudol, M., and Kelly, J. W. (1999) WW: An isolated three-stranded antiparallel β-sheet domain that unfolds and refolds reversibly; evidence for a structured hydrophobic cluster in urea and GdnHCl and a disordered thermal unfolded state. *Protein Sci. 8*, 841–853.
39. Martinho, J. M. G., Santos, A. M., Fedorov, A., Baptista, R. P., Taipa, M. A., and Cabral, J. M. S. (2003) Fluorescence of the single tryptophan of cutinase: Temperature and pH effect on protein conformation and dynamic. *Photochem. Photobiol. 78*, 15–22.
40. Petersen, M. T. N., Johnson, P. H., and Petersen, S. B. (1999) Amino acid neighbors and detailed conformational analyses of cysteines in proteins. *Protein Eng. 12*, 535–548.
41. Longhi, S., Czjzek, M., Lamzin, V., Nicolas, A., and Cambillau, C. (1997) Atomic resolution (1.0 Å) crystal structure of *Fusarium solani* cutinase: Stereochemical analysis. *J. Mol. Biol. 268*, 779–799.
42. Kim, S. W., Joo, S., Choi, G., Cho, H.-S., Oh, B.-A., and Choi, K. Y. (1997) Mutational analysis of the three cysteines and active site aspartic acid 103 of ketosteroid isomerase from *Pseudomonas putida* biotype B. *J. Bacteriol. 179*, 7742–7747.
43. Hutchison, R. S., Betts, S. D., Yocum, C. F., and Barry, B. A. (1998) Conformational changes in the extrinsic manganese stabilizing protein can occur upon binding to the photosystem II reaction center: An isotope editing and FT-IR study. *Biochemistry 37*, 5643–5653.
44. Vincenzetti, S., Cambi, A., Maury, G., Bertorelle, F., Gaubert, G., Neuhard, J., Natalini, P., Salvatori, D., De Sanctis, G., and Vita, A. (2000) Possible role of two phenylalanine residues in the active site of human cytidine deaminase. *Protein Eng. 13*, 791–799.
45. Pantazatos, D. P., and MacDonald, R. I. (1997) Site-directed mutagenesis of either the highly conserved Trp-22 or the moderately conserved Trp-95 to a large, hydrophobic residue reduces the thermodynamic stability of a spectrin repeating unit. *J. Biol. Chem. 272*, 21052–21059.
46. Markovic-Housley, Z., Stolz, B., Lanz, R., and Erni, B. (1999) Effects of tryptophan to phenylalanine substitutions on the structure, stability, and enzyme activity of the IIAB(Man) subunit of the mannose transporter of *Escherichia coli*. *Protein Sci. 8*, 1530–1535.
47. Feinberg, B. A., Petro, L., Hock, G., Margoliash, E., and Qin, W. (1999) Linking entropies of reaction to protein stability: Tyr-67-Phe variants of two cytochromes c. *J. Inorg. Biochem. 74*, 126.

BI800225M