Mutagenesis of Basic Residues R151 and R161 in Manganese-Stabilizing Protein of Photosystem II Causes Inefficient Binding of Chloride to the Oxygen-Evolving Complex†

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ABSTRACT: Manganese-stabilizing protein of photosystem II, an intrinsically disordered polypeptide, contains a high ratio of charged to hydrophobic amino acid residues. Arg151 and Arg161 are conserved in all known MSP sequences. To examine the role of these basic residues in MSP structure and function, three mutants of spinach MSP, R151G, R151D, and R161G, were produced. Here, we present evidence that replacement of Arg151 or Arg161 yields proteins that have lower PSII binding affinity, and are functionally deficient even though about 2 mol of mutant MSP/mol PSII can be rebound to MSP depleted PSII membranes. R161G reconstitutes O_2 evolution activity to 40% of the control, while R151G and R151D reconstitute only 20% of the control activity. Spectroscopic and biochemical techniques fail to detect significant changes in solution structure. More extensive O_2 evolution assays revealed that the Mn cluster is stable in samples reconstituted with each mutated MSP, and that all three Arg mutants have the same ability to retain Ca^{2+} as the wild-type protein. Activity assays exploring the effect of these mutations on retention of Cl^-, however, showed that the R151G, R151D, and R161G MSPs are defective in Cl^- binding to the OEC. The mutants have Cl^- K_M values that are about four (R161G) or six times (R151G and R151D) higher than the value for the wild-type protein. The results reported here suggest that conserved positive charges on the manganese-stabilizing protein play a role in proper functional assembly of the protein into PSII, and, consequently, in retention of Cl^- by the O_2-evolving complex.

Water oxidation is the reaction by which all photosynthetic organisms produce and release O_2 into the atmosphere. This reaction takes place in the O_2-evolving complex (OEC[1]) of photosystem II (PSII). The OEC consists of several inorganic cofactors (Ca^{2+}, Cl^-, and four Mn atoms), and three extrinsic polypeptides (33, 23, and 17 kDa) that are attached to the intrinsic subunits of PSII (*1*). Proper orientation and mutual coordination of these components are important for formation of the active site of the OEC. In most eukaryotes, the 23 and 17 kDa extrinsic polypeptides are required for high affinity binding of Cl^- and Ca^{2+} (*1*). The 33 kDa extrinsic protein, also known as the manganese-stabilizing protein (MSP), also plays a role in facilitating binding of Ca^{2+} and Cl^- to PSII, which promotes high rates of O_2 evolution. Several studies have demonstrated that high concentrations of Ca^{2+} and Cl^- provide a partial substitution for MSP; in the presence of unphysiologically high Cl^- concentrations, MSP-depleted PSII membranes retain an intact Mn cluster, and low rates of O_2 evolution are observed if high concentrations of Cl^- and Ca^{2+} are present in the assay medium (*2*, *3*). MSP is therefore necessary for high rates of O_2 evolution and for stability of the Mn cluster, but does not appear to participate directly in the mechanism of the O_2-evolving reaction. Although it has been suggested that the 33 kDa protein is not involved directly or indirectly in binding of Ca^{2+} in the OEC (*4*), a number of studies indicate that MSP is required for retention of Ca^{2+} in PSII (*5*−*7*). This is proposed to be due either to direct ligation of the metal by MSP or indirectly through interactions between MSP and the subunit to which Ca^{2+} is bound. For Cl^-, it has been hypothesized that MSP might provide a ligand to the anion during the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions, maintain the integrity of associated proteins to which Cl^- is ligated, or simply trap free Cl^- in close proximity to the Mn cluster (*8*).

The ability of MSP to fulfill its function in the OEC is related to its natively unfolded or intrinsically disordered

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[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; DCBQ, 2,6-dichloro-1,4-benzoquinone; IPTG, isopropyl-β-Δ-thiogalactopyranosidase; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein; MRE, mean residue ellipticity; OEC, oxygen-evolving complex; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; *psbO*, gene encoding precursor MSP; SDS, sodium dodecyl sulfate; sw-PSII, NaCl-washed photosystem II membranes depleted of 23 and 17 kDa extrinsic polypeptides; TMACl, tetramethylammonium chloride; usw-PSII, urea NaCl-washed photosystem II membranes depleted of 33, 23, and 17 kDa extrinsic polypeptides; UV, ultraviolet.

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behavior in solution. Natively unfolded proteins are often important components of multisubunit complexes. In solution, their secondary structure contains an unusually high content of random coils and turns, their amino acid sequences are composed of a high ratio of charged to hydrophobic residues, and such proteins exhibit anomalous mobility on SDS−PAGE, a highly acidic or basic p*I*, and thermostability, as evidenced by their ability to regain solution structure and activity after heating (*9*−*11*). In the case of MSP, its binding to PSII is proposed to occur by a two step mechanism that hypothesizes that the protein binds, and then refolds into a structure that is presumably more compact and which allows for binding of other extrinsic PSII subunits, and retention of the essential inorganic cofactors (*12*). The N-terminal amino acid residues Thr7 and Thr15 (spinach numbering) are essential for docking of spinach MSP to PSII (*13*). Functional refolding of this precursor form of MSP to the more tightly bound conformation requires the presence of the C-terminal residues Leu245 and Gln244 (*14*). A number of studies on MSP in solution provide evidence that the protein's N- and C-termini are in proximity to one another, and that alterations in these domains significantly influence this interaction (*15*, *16*). The conserved disulfide bridge in spinach MSP, between Cys28 and Cys51, is not required for MSP function or for its thermostability (*17*). Experiments such as dynamic light scattering, ultracentrifugation, or low angle X-ray scattering provide evidence that MSP has a prolate ellipsoid shape (*18*, *19*). Biochemical data on both prokaryotic and eukaryotic MSPs show that the protein in solution has more than 50% random coils and turns; β-sheets (about 35%) comprise the dominant component of secondary structure. Crystallographic data on cyanobacterial PSII at 3.2−3.8 Å have provided the basis for models showing the structure of the PSII-bound form of MSP. The models predict that it has a barrel-like shape with the N-terminus protruding on one side of the barrel toward the CP43 polypeptide. The other side of the barrel has a protruding unordered loop pointing toward the E loop of the CP47 polypeptide (*20*−*23*). This loop in the MSP structure is the domain that is closest to the OEC Mn cluster (*22*); the model in ref *22* proposes that MSP contributes no ligands to Ca^{2+}.

A number of studies have shown that the primary amino acid sequences of MSPs from various photosynthetic organisms are homologous (*24*, *25*). One of the most conserved domains is the region Phe145−Gly163 (spinach numbering) that includes two fully conserved arginyl residues (R151 and R161) in all known MSP sequences. When R152 and R162 from *elongatus*, which are homologous to R151 and R161 in spinach, were mutated (*25*), the resulting proteins were found to possess defects in PSII binding and in reconstitution of O_2 evolution activity. These mutants also exhibited nonspecific binding to PSII, but their solution structures were unchanged. To determine the structural and functional importance of these residues in spinach MSP, site-directed mutagenesis was employed to produce mutants R151G, R151D, and R161G, which either eliminate one positive charge or convert a positive charge to a negative charge. The data presented here show that all mutants are deficient in binding to PSII, as compared to wild type, and that their ability to reconstitute O_2 evolution is likewise impaired even though all three mutants will rebind about 2 copies per PSII reaction center. Characterization of the solution structures of the mutant proteins by spectroscopic and biochemical techniques fails to detect any significant changes compared to the wild-type protein. Results from reconstitution of usw-PSII membranes with mutants in the presence of varying Ca^{2+} or Cl^- concentrations indicate that MSP is involved in maintenance of Cl^- in proximity to the Mn cluster. It is hypothesized that removal of R151 and R161 from the MSP sequence could impair functional refolding of the protein after its initial docking to PSII, which could in turn lead to less efficient binding of Cl^- to the OEC.

MATERIALS AND METHODS

Mutations of the psbO gene and Transformation of Escherichia coli Cells. The *psbO* gene from spinach carrying the R151D mutation was produced as described in ref *17*, except that other synthetic oligonucleotides were used for mutagenesis; the codon specifying R151 was changed to the codon for D151. The plasmid construct *psbO*-TOPO (Invitrogen) was used for the R151G and R161G mutants. Mutations were inserted into the construct using a Stratagene mutagenesis technique and synthetic oligonucleotides (QuickChange XL II site-directed mutagenesis kit; Stratagene, La Jolla, CA). The codons for R151 or R161 were changed to those for G151 or G161. Insertion of the correct mutations and an absence of errors in the *psbO* sequence were confirmed by DNA sequencing, and the mutated *psbO*-TOPO constructs were transformed into BL21(DE3)pLysS *E. coli* cells. The correct reading frame (starting with EGGKR) during protein translation and subsequent processing in *E. coli* was verified by Edman degradation of all three purified mutant proteins.

*Overexpression and Purification of Recombinant MSP*s. The R151D mutant was overexpressed and purified as described in ref *17*, while the R151G and R161G MSPs were produced by overexpression of the *psbO*-TOPO vector for 3.5 h in LB media containing 50 μg/mL ampicillin and 25−32.5 μM IPTG. Purification of these proteins was carried out as in ref *13* except that a second purification step (a step gradient of 30 mM, 150 mM, 500 mM, and 1 M NaCl) was applied after linear gradient elution of MSP to further purify the protein, which was eluted at 150 mM NaCl. Both chromatographic procedures yield pure, soluble protein.

Reconstitution of PSII with Recombinant MSP. Intact PSII, sw-PSII, and usw-PSII membranes were prepared from spinach as described in ref *26* and stored in 0.4 M sucrose, 50 mM MES (pH 6) and 200 mM NaCl, to provide optimum stability to the OEC (*2*, *3*, *24*, *26*). No loss of Mn from PSII is observed under these conditions of storage (data not shown). For the reconstitution experiments shown in Figure 1, usw-PSII membranes were reconstituted with MSP in a solution containing 37 mM MES (pH 6), 10 mM $CaCl_2$, 70 mM NaCl, 100 μg BSA/mL, 0.3 M sucrose, and 2% betaine (w/v). The Chl concentration was 200 μg/mL. Oxygen evolution assays were performed with DCBQ (600 μM), as the artificial acceptor, using SMTC buffer (0.4 M sucrose, 50 mM MES (pH 6), 20 mM $CaCl_2$, and 60 mM TMACl) containing 100 μg of BSA/mL; the Chl concentration was 10 μg/mL. For experiments to examine the stability of the Mn cluster (Table 1), reconstitution and activity assays were done in the same way as those described above, except that 10 mM $CaCl_2$ was replaced by 10 mM $Ca(MES)_2$, and NaCl

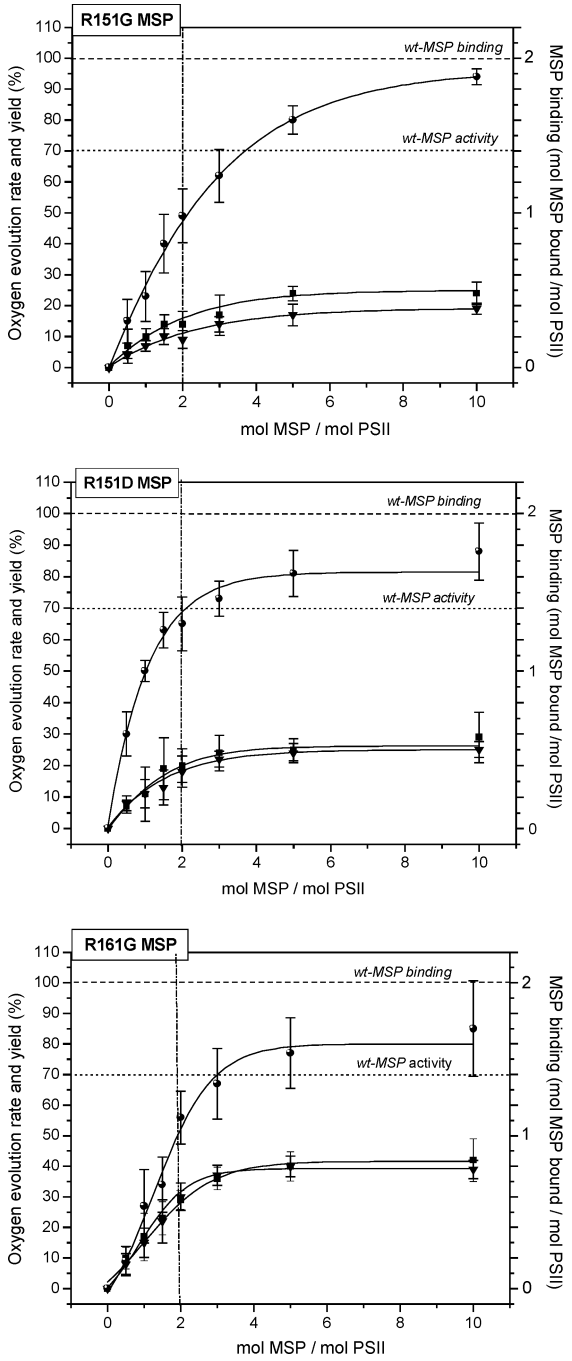

FIGURE 1: Recovery of O_2 evolution activity by usw-PSII membranes reconstituted with R151G, R151D, and R161G MSP, respectively. Circles: binding curves. Squares: O_2 evolution rate. Triangles: O_2 evolution yield plotted as a function of moles of MSP added to reconstitution mixtures. Oxygen rates and yields were assayed for 1 and 4 min of continuous illumination, respectively. Samples were reconstituted for 1 h at room temperature. Residual activity of usw-PSII was subtracted from the activities of reconstituted samples. 100% of activity corresponds to the activity of sw-PSII sample (300−400 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}). Points are averages of 3−5 independent experiments, and the vertical bars at each point give the standard deviation. Dotted, dashed, and dotted-dashed lines show saturation of the wild-type MSP activity and binding at 2 mol of MSP/mol of PSII.

was added to obtain 20 or 100 mM Cl^- in the reconstitution mixture. To determine the Ca^{2+} and Cl^- K_M values, O_2 evolution activity of the reconstituted samples was measured with varying concentrations of Ca^{2+} or Cl^- (Table 2). The reconstitution mixture for the Cl^- experiment contained 10 mM Ca^{2+} and 20 mM Cl^-, while the reconstitution mixture for the experiment with Ca^{2+} contained 10 mM Ca^{2+} and 110 mM Cl^-. The dependence of O_2 evolution activity of reconstituted samples on the Cl^- concentration was performed under the same conditions as those described above, except that Cl^- concentrations ranged from 800 μM to 100 mM; Ca^{2+} was held constant at 20 mM. The increase in O_2-evolving activity of reconstituted samples by added Ca^{2+} was measured at a constant Cl^- concentration (100 mM), the Ca^{2+} concentration ranging from 400 μM to 20 mM. The extent of rebinding of the recombinant protein to usw-PSII was determined by SDS−PAGE. To eliminate the possibility of binding artifacts related to unbound MSP, reconstitution mixtures were centrifuged (12000*g* for 10 min at 4 °C). The pellet was resuspended in 800 μL of the SMTC buffer and centrifuged again at 4 °C for 10 min. This pellet was resuspended in 60 μL of SMTC to a concentration of 1 mg of Chl/mL. The UN-SCAN-IT program was used for estimation of the amount of MSP bound to PSII by integration of the Coomassie-stained MSP bands. A sw-PSII sample was used as the 100% control. The Coomassie-stained 47 kDa protein was used as an internal standard to correct for errors in loading protein samples on the gel.

Table 1: O_2 Evolution Activity of sw-PSII Membranes and usw-PSII Membranes Reconstituted with the R151G or R161G Mutant MSP[a]

	oxygen evolution activity (μmol of O_2/mg of Chl/h)							
	20 mM Cl^-				100 mM Cl^-			
time (h)	sw-PSII	usw-PSII	R151G	R161G	sw-PSII	usw-PSII	R151G	R161G
0	334	88	160	224	374	104	160	240
2	366	96	160	248	358	120	176	224
4	334	88	168	208	358	104	176	216
6	334	80	152	216	350	80	184	224
21	318	88	136	216	358	96	176	200

[a] After reconstitution of usw-PSII with MSP (9 mol of MSP/mol of PSII) at 20 mM chloride or 100 mM chloride at room temperature, oxygen evolution rate was measured by taking aliquots during incubation of samples on ice over a time period of 21 h.

Table 2: K_M Values for sw-PSII Preparations (Containing Wild-Type MSP) and usw-PSII Preparations Reconstituted with the R151G, R151D, or R161G Mutant MSP (10 mol of MSP/mol of PSII)[a]

	K_M (mM)	
sample	Cl^-	Ca^{2+}
sw-PSII	0.41	0.27
usw-PSII + R151G	2.60	0.22
usw-PSII + R151D	2.20	0.17
usw-PSII + R161G	1.50	0.22

[a] Constants were determined from O_2 evolution rate plotted against increasing concentration of calcium or chloride.

UV and CD Spectroscopy and Size-Exclusion Chromatography. Recombinant MSPs were dialyzed in 10 mM KH_2PO_4 buffer (pH 6) and used for spectroscopic experiments as described in refs *13* and *26*. Mutant proteins in SMN buffer (0.4 M sucrose, 50 mM MES (pH 6), and 10 mM NaCl) were used for gel filtration experiments as described in ref *26*. Molecular mass values are reported as the average of 4 or 5 experiments for each mutant with maximum error of ±1.3 kDa.

RESULTS

Functional Integrity of R151G, R151D, and R161G MSP Mutants. R151 and R161 are fully conserved amino acid residues located near the middle of the primary sequence of MSP. Figure 1 presents data on reconstitution of usw-PSII membranes with the three Arg mutants. Binding curves (circles) show that all of the mutants had lower PSII binding affinities than did wild type. At 2 mol of MSP/mol of PSII, 1−1.3 mol of mutant protein was bound to the usw-PSII preparations, as compared to the 2 mol of wild-type protein that rebinds to usw-PSII, indicated by the dashed lines for 100% activity in the figure. When higher protein concentrations were used in reconstitution experiments, gradual saturation of binding was observed; maximum binding to the usw-PSII samples used in these experiments occurred at about 2 mol of MSP bound, after incubation of 10 mol of an Arg mutant MSP/mol of PSII in a reconstitution mixture. None of the mutant proteins exhibited nonspecific binding when this was assayed using intact PSII membranes (see ref *13*, data not shown). The O_2 evolution assays presented in Figure 1 show that even though up to 2 mol of mutant MSPs can be rebound to PSII, none of these mutant proteins are capable of restoring high levels of activity. The R161G mutant reconstituted about 40% of the control O_2 evolution activity, while R151G and R151D restored only about 20% of the control activity (Figure 1). The activity of a sw-PSII preparation assayed under the same conditions was used as the control. None of the mutants were cold-sensitive (data not shown), a phenomenon previously observed with the MSP mutant V235A (*27*). The binding and functional behaviors of these mutants are somewhat similar to those of two C-terminal mutants of MSP, L245E, and L245@ (which replaces the leucine codon with a stop codon). These C-terminal mutants have a greatly expanded solution structure that suggests that they are less compact in solution than is the wild type (*14*). To examine the possibility that a modified solution conformation is responsible for the defect created by these Arg mutants, the solution structures of R151G, R151D, and R161G were examined more closely.

Structural Characterization of Arg Mutants. Gel filtration analysis of MSP and its several mutants has shown that the wild-type protein exhibits a much larger (37−40 kDa) molecular mass than would be predicted from its amino acid composition (26.5 kDa). When this analysis was applied to the Arg mutants, it was found that R151G, R151D, and R161G exhibited apparent molecular masses of 36.1, 36.3, and 35.7 kDa, respectively, which is similar to the apparent mass of wild-type MSP (*26*), indicating that site-directed mutagenesis of these Arg residues did not cause a drastic change in the overall size of the protein.

The UV-absorption spectra of proteins can monitor the local environment of aromatic amino acid residues. Modification of the hydrophobicity of this environment is manifested as an alteration in the amplitude, or as a blue shift of the characteristic absorption peaks. Among the features of the UV spectrum of wild-type MSP are a shoulder due to Trp at 293 nm, a Tyr peak at 276 nm, and two small, broad peaks at 259 and 266 nm arising from Phe (*14*). Any significant change in the mutant's solution structure could affect these spectral features. Figure 2A shows UV spectra of wild-type MSP and the Arg mutants. The similar position and amplitude of the peaks in the spectra shown here indicate that the local environment of aromatic amino acids in the mutants has not been altered.

Wild-type MSPs from spinach and other eukaryotes have a single Trp residue at position 241 in their primary sequences; this residue is buried in a hydrophobic environment (*12*). Near-UV CD spectroscopy monitors changes in this domain at the MSP C-terminus, because Trp241 generates a major peak at 292 nm in the near-UV CD spectrum of wild-type MSP. In theory, the eight Tyr of spinach MSP can contribute to the spectrum's second major peak at 285 nm, but only those residues that are in hydrophobic environments make major contributions to the near-UV CD spectral peak. Phe peaks, centered at 258 and 266 nm, are less pronounced than the Tyr and Trp signals in the wild-type near-UV CD spectrum, and originate from the Phe residues distributed over the MSP domains that are solvent inaccessible (*12*). Comparison of near-UV CD spectra of wild type with the near-UV CD spectra of R151G, R151D, and R161G would detect structural changes that occur mainly in the C-terminal hydrophobic region of the protein. Figure 2B presents near-UV CD spectra of wild-type MSP and of the Arg mutants. As can be seen, the spectra of wild type and mutant MSPs are very similar, indicating that the tertiary structural elements of the mutants that are monitored by these CD signals have not changed compared to the wild-type protein (Figure 2B). The secondary structure content of the mutants was characterized by far-UV CD spectroscopy (see ref *26*, data not shown). The results of these experiments indicate that there have been no significant changes in the secondary structure (α-helix, β-sheet) contents of the mutants, relative to the contents of wild-type MSP. Taken together, the results presented here suggest that, in solution, R151G, R151D, and R161G retain the secondary and tertiary structural characteristics of the wild-type MSP.

The Mn Cluster Remains Stable in Reconstituted Samples. In the absence of MSP, high (>100 mM) Cl^- concentrations stabilize the PSII Mn cluster and O_2 evolution occurs, although at a lower rate (*28*). For the experiments shown in Figure 1, examination of the functional behavior of mutant MSPs rebound to usw-PSII membranes was carried out in the presence of 110 mM Cl^-. Nevertheless, the binding curves shown in Figure 1 indicate that Arg mutants have lowered PSII binding affinity compared to the wild-type protein. This loss of O_2 evolution activity in samples reconstituted with the Arg mutants could therefore be interpreted as the consequence of disintegration of the Mn cluster, combined with a slow loss of Mn^{2+} atoms from the OEC. To test this hypothesis, usw-PSII reconstituted with R151G or R161G at 20 mM or 100 mM Cl^- concentrations were placed on ice in the dark and assayed for O_2 evolution activity over a period of 21 h. The activities of sw- and usw-PSII membranes were taken as the controls. If the low PSII binding affinity of Arg mutants is responsible for release of Mn atoms from the OEC that leads to a decrease in activity, samples reconstituted at a low Cl^- concentration should exhibit a decrease in activity after incubation on ice for several hours, when compared to samples reconstituted with high concentrations of Cl^-. The data in Table 1 show no significant decrease in activity of any samples reconstituted with R151G or R161G mutant proteins at 20 mM Cl^-, compared to 100 mM Cl^-. The same result was obtained

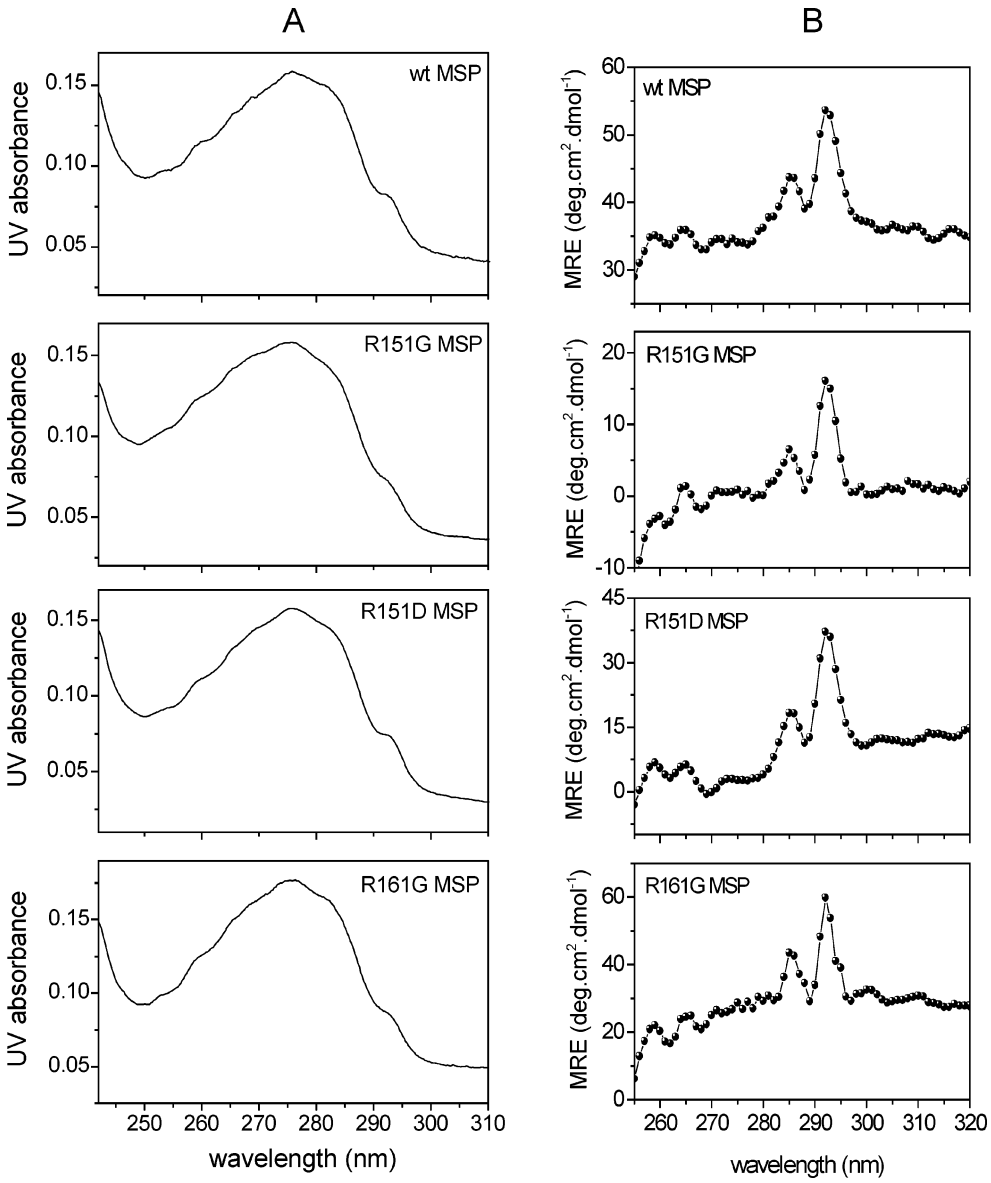

FIGURE 2: (A) UV-absorption spectra of wild-type MSP, R151G MSP, R151D MSP, and R161G MSP. Spectra are shown for 10 μM protein in 10 mM KH_2PO_4 buffer, pH 6. The experimental conditions were as follows: path length, 1 cm; temperature, 25 °C; sample volume, 1 mL; scan width, 310−240 nm. (B) Near-UV CD spectra (protein solution minus buffer) of wild-type MSP, R151G MSP, R151D MSP, and R161G MSP. Spectra are recalculated on the basis of the protein concentration. The experimental conditions were as follows: path length, 1 cm; temperature, 25 °C; sample volume, 1 mL; scan width, 320−250 nm; time constant, 1 s; bandwidth, 1 nm.

with R151D (data not shown). As the table shows, a usw-PSII sample, depleted of MSP, also retains activity in the presence of 20 mM Cl^- over the same time period. This result is different from that obtained by Miyao and Murata (*28*), who observed a decrease in O_2 evolution activity of MSP-depleted PSII samples after 4 h of dark incubation. However, these authors used 10 mM rather than 20 mM Cl^- in their experiments to test the stability of the Mn cluster. An attempt was made to lower the Cl^- concentrations in PSII samples, but the washing step required to accomplish Cl^- depletion resulted in activity losses in all samples. When an experiment was carried out in which the usw-PSII membranes were diluted with 400 mM sucrose and 50 mM MES (pH 6) to attain a final Cl^- concentration of 10 mM, an inactivation of usw-PSII membranes was observed over a 6-h period, in agreement with the results reported by Miyao and Murata (*28*). Therefore, the results presented in Table 1 indicate that the activity reconstituted by the recombinant mutant MSP proteins, although lower than the control, is stable for long periods of time at a Cl^- concentration at or near the minimum required to stabilize the OEC. It is therefore likely that destabilization of the OEC Mn cluster is not responsible for the decreased activity of PSII samples reconstituted with the mutant proteins.

Reconstitution and Activity Assays with Varied Concentrations of Ca^{2+} or Cl^-. The effect of Arg mutations on retention of Ca^{2+} and Cl^- was also characterized in experiments where usw-PSII membranes were reconstituted with R151G, R151D, or R161G MSPs, and O_2 evolution was assayed in the presence of varying concentrations of Ca^{2+} or Cl^- (for experimental details, see Materials and Methods). In the case of Ca^{2+}, all samples assayed have about the same requirement for the metal, although the data in Table 2 show that the Ca^{2+} K_M is slightly lower for the mutants than it is for the wild-type protein. This phenomenon can be explained by removal of a positive charge from the MSP sequence. A

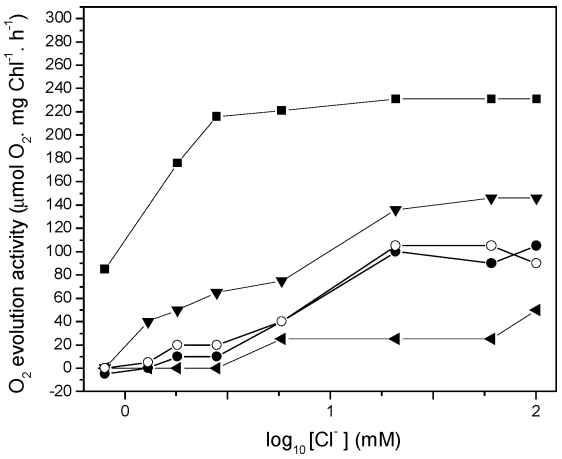

FIGURE 3: O_2 evolution activity of sw-PSII membranes (■), usw-PSII membranes (solid sideways triangle), and usw-PSII membranes reconstituted with R151G MSP (●), R151D MSP (○), or R161G MSP (▼). Activity is plotted as a logarithmic function of increasing concentrations of chloride. The Ca^{2+} concentration was held constant at 20 mM. Samples were reconstituted with 20 mM Cl^- for 1 h at room temperature. Points are the averages of two measurements.

different result was obtained, however, when the effect of Cl^- was investigated. Data in Figure 3 show that wild-type MSP has a Cl^- K_M of 0.41 mM (see Table 2). The Arg mutants, on the other hand, require more than 10 mM Cl^- to reach maximal rates of O_2 evolution (Figure 3). The R161G mutant has a Cl^- K_M almost four times higher than wild type (1.5 mM). Mutants R151G and R151D have K_M values about six times higher than the wild type (2.60 mM and 2.20 mM, respectively), and they exhibit similar responses to increasing concentrations of Cl^- (Figure 3 and Table 2). These results indicate that the mutations at R151 or R161 produce MSP species whose defect in binding to PSII also produces a defect in Cl^- binding to the OEC.

DISCUSSION

Manganese-stabilizing protein contains a large number of conserved positively or negatively charged amino acid residues, a feature that is one of the characteristics of natively unfolded, or intrinsically disordered proteins (*9*−*11*). The charged amino acid residues in MSP could have several functional and structural roles. Repulsion forces between negatively charged residues on the protein surface might prevent aggregation under conditions where MSP would be present at high concentration in the chloroplast lumen, where the pH is near the p*I* of MSP (pI = 5.2 (*29*)). Both positive and negative charges in MSP have been identified as potential participants in the electrostatic intra- or intermolecular interactions within MSP or between MSP and PSII (*24*, *30*, *31*). The charged residues D158, D222, D223, D224, H228, and E229 of MSP (*Thermosynechococcus elongatus* numbering) have been proposed to participate in a hydrophilic proton channel between the OEC and the thylakoid lumen (*32*).

In this work we present data on a functional role of the conserved Arg151 and Arg161 residues in spinach MSP. Our results show that Gly or Asp mutations in R151 or a Gly mutation in R161 affects the protein's binding affinity for PSII, as well as its ability to restore O_2 evolution activity. Binding of about 2 mol of a mutant MSP to PSII required almost 10 mol of MSP/mol of PSII in reconstitution mixtures, and no nonspecific binding to PSII was observed. When about 2 mol of the protein was bound to PSII, R161G restored 40% and R151G and R151D reactivated only 20% of the control activity. This functional behavior is somewhat different from what was observed with Arg mutants of cyanobacterial MSP by Motoki et al. (*25*). They reported that the *S. elongatus* mutations in R152 (R152Q) and R162 (R162Q) (which are homologous to spinach residues R151 and R161) caused lower PSII binding affinity, but that these proteins also exhibited significant nonspecific binding to PSII. Both mutants reconstituted 16−18% of the control activity (*25*). Comparison of the activities reconstituted by the mutants described in this study with those reported in ref *25* reveals that the R162Q mutation in cyanobacterial MSP had a greater deleterious effect on activity than did R161G in spinach MSP. This would indicate that disruption of the single copy of MSP that is present in prokaryotes (*20*−*23*) is more critical than is the defect caused by the same mutation in spinach PSII, where the experimental evidence supports the presence of two rather than one copy of MSP (*33*−*35*). In the case of the Arg residues targeted for mutagenesis in this work, the corresponding Arg residues, R152 and R162 of *T. elongatus* MSP, are positioned about 8−10 Å from Glu 310 and Glu 307 or Asp308 of D2, respectively, and R152 is about 5 Å from E65 on D1, according to the crystal structure of Ferriera et al. (*22*, *32*). If these distances are confirmed by higher resolution structures, then it is unlikely that either of these arginyl residues is involved in H-bonding or salt-bridge interactions with the intrinsic subunits of PSII. It is possible, given the presence of two copies of MSP in higher plant PSII (*33*−*35*), that different functional responses to the replacement at a conserved Arg site in prokaryotic and eukaryotic MSP could be a consequence of differences either in prokaryotic and eukaryotic organization of PSII and/or of some subtle structural differences between prokaryotic and eukaryotic MSP.

When the spinach MSP mutants were examined for structural alterations, none of the methods used revealed modifications to secondary structure or to the elements of tertiary structure that can be monitored by UV−vis or near-UV CD spectroscopies. Gel filtration results are consistent with the spectroscopic data. These results are in agreement with the conclusions of Motoki et al. (*25*), who found that the near- and far-UV CD spectra of the R152Q and R162Q mutants of *S. elongatus* were similar to those of the wild-type protein. Thus, if mutagenesis of the homologous Arg residues in spinach or cyanobacterial MSP generates a structural change in the proteins' solution conformations, this change must be relatively subtle to escape detection by the techniques used here.

For *S. elongatus* MSP, it was proposed that R152 and R162 participate directly in an interaction with PSII (*25*), possibly in a charge-pair interaction, and that this is responsible for the observed disruption of activity associated with these mutants. Such interactions are not apparent in the structure of *T. elongatus* PSII at the current resolution (*22*), and it is also unclear from the crystal structure why these cyanobacterial Arg mutants would cause nonspecific binding of MSP to PSII. Because the data on the spinach MSP Arg mutants show only less efficient, rather than nonspecific, binding, it is possible that the defect in activity in the spinach

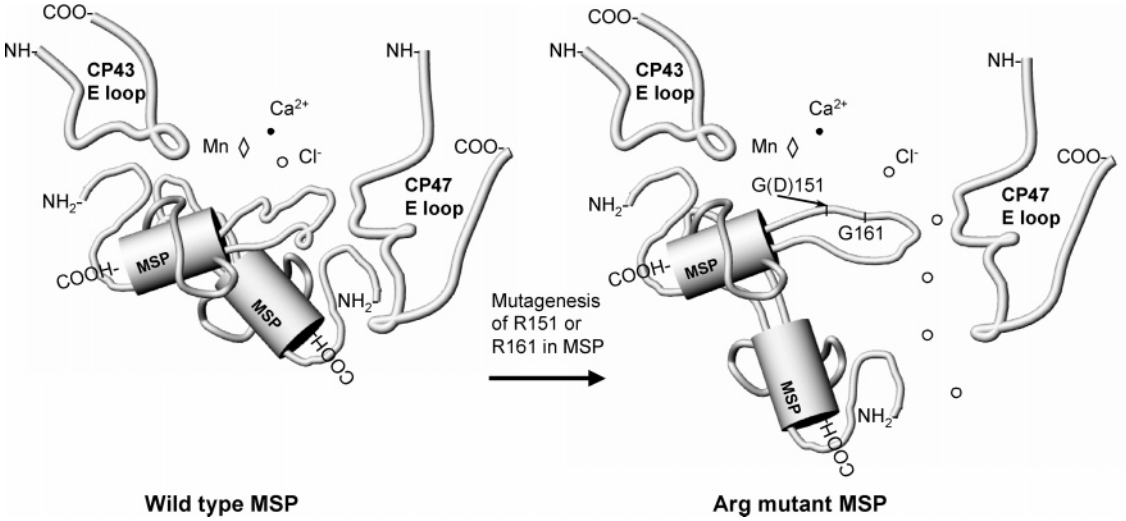

FIGURE 4: Hypothetical model of the CP43−MSP−CP47 interface in spinach PSII, showing wild-type MSP (left) and MSP Arg mutants (right) (barrels with flexible curves) bound to the CP43 and CP47 E loops. The model proposes that mutations in R151 or R161 induce a defect in proper refolding of MSP when it binds to PSII, which results in lower affinity binding of these proteins to their sites in PSII. This defect in binding and refolding could lead to lower retention of chloride in the vicinity of the Mn cluster. Positions of the mutated residues G(D)151 and G161 in MSP are shown in the model on the right. Mn cluster (◊); Ca^{2+} (●); Cl^- (○). Graphics were constructed using the Rhinoceros 3.0 (evaluation version) and MS PowerPoint 2003 programs.

system is linked to modified binding to PSII of one or more of the inorganic cofactors, Mn, Ca^{2+}, or Cl^-, of the OEC. The results of the experiments presented in Tables 1 and 2 and Figure 3 indicate that none of the Arg mutations had a detectable effect on Mn binding or on retention of Ca^{2+}; instead, Cl^- retention by the OEC is negatively affected. Determination of the Cl^- K_M value for the R161G mutant gives a value about four times higher than wild type, and the R151G and R151D mutants have K_M values about six times higher than that of wild type. These results clearly indicate that replacement of arginyl residues with Gly or Asp in spinach MSP has an effect on the protein's ability to facilitate retention of Cl^- in the OEC. The fact that relatively minor perturbations of Ca^{2+} binding are observed in the presence of rather substantial changes in the Cl^- K_M with these mutants would suggest that the binding modes of the two cofactors must be different. This is consistent with the isotope labeling experiments of Lindberg et al. (*36*) and Adelroth et al. (*37*), who found that removal of the 23 and 17 kDa polypeptides caused rapid exchange of Cl^-, whereas Ca^{2+} could be retained by PSII. Removal of MSP was required to abolish the PSII Ca^{2+} binding site, an observation that was attributed either to an effect on the binding site itself or, alternatively, to removal of a diffusion barrier between the Ca^{2+} site and the bulk medium.

Mutagenesis of basic amino acid residues in the sequence of the large extrinsic "E" loop of CP47 of *Synechocystis 6803* also affects Cl^- retention by the OEC. Putnam-Evans et al. (*38*, *39*) examined site-directed mutants R448G and K321G of CP47, and Tichy and Vermaas (*40*) studied combinatorial mutants in the region D440−P447 of CP47. The results reported in refs *38* and *39* showed that, in the presence of standard Cl^- concentrations used to culture wild-type cells, R448G grew at a rate that was about 50% of the control, while the K321G mutant strain exhibited the same growth rate as the control strain. Neither mutant was, however, able to grow photoautotrophically under Cl^- limiting conditions. Under these conditions, R448G failed to assemble PSII centers, while K321G assembled about 50% of the functional PSII centers found in the wild-type strain. For both mutants, the authors hypothesized either that the basic residues R448 and K321 form a Cl^- binding domain close to the OEC active site or, alternatively, that mutations in these residues induce a conformational change in CP47 that disrupts a distant Cl^- binding site (*38*, *39*). Mutants reported in ref *40* carrying either deletion or random mutation in the D440−P447 region of CP47 grew poorly under Cl^- limiting conditions, and exhibited photoinhibition under normal growth conditions. An increasing concentration of Cl^- in the growth medium could, to some extent, compensate the Cl^- requirement. The authors of this study suggested that these mutants were deficient in Cl^- binding (*40*). The basic residues K321 and R448, as well as the region enclosed by D440−P447, are localized in the large extrinsic E loop of CP47 (*41*). In spinach PSII, this loop was shown to interact with a domain of MSP that includes the N-terminus (*42*). The cyanobacterial crystal structure, on the other hand, predicts an interaction of the E loop of CP47 with the flexible loop of MSP that is localized near the middle of its primary sequence (*22*). If the mutations in the E loop of CP47 that are described in refs *38* and *39* induced a conformational change in its structure, this change could in theory disrupt an interaction with MSP.

Upon binding to PSII, MSP is predicted to gain additional β-sheet structure (*43*), and therefore it has been proposed (*12*, *14*) that MSP interacts with PSII in a two-step mechanism. According to this proposal, MSP first binds to PSII and then, in a second step, undergoes some refolding at its binding sites on PSII. It is possible that the refolding of MSP that creates the tightly bound form of the protein also facilitates formation of a structure that facilitates Cl^- retention (see Figure 4, wild type). The alternative, that removal of a single positive charge on MSP disrupts an electrostatic interaction between the protein and Cl^-, seems unlikely, because the experimental evidence assigns the Cl^- binding site at or near the Mn cluster (*44*). It also seems unlikely that Arg residues on MSP or on CP47 that are separated from one another could collectively form a binding

site for the one Cl^- that is associated with the active site of the OEC in such a way that mutagenesis of one Arg residue (this work, *38*, *39*) could affect the site that binds the one Cl^- atom that was detected in the OEC active site (*45*). We would propose a hypothetical model to describe events at the CP43−MSP−CP47 interface after the replacement of R151 or R161 in spinach MSP, which is shown schematically in Figure 4. The Arg mutants retain the wild-type solution structure, and their MSP N-termini, a determinant for the first binding step (*13*), remain intact. Because they exhibit a lower PSII binding affinity than does the wild type, we hypothesize that replacement of arginyl residues in spinach MSP could impair the second step of the MSP−PSII binding and assembly process, fully functional refolding of the protein after its docking to PSII. As a result, improperly refolded MSP could lead to less efficient retention of Cl^- in the O_2-evolving complex (Figure 4, Arg mutants). The similar defect that is induced by insertion of mutations into the E loop of CP47 (*38*, *39*) may also arise from defective MSP assembly. Taken together, a disruption of the MSP−CP47 interface by mutation in either of the interacting proteins is likely to cause inefficient binding of Cl^- to the OEC active site.

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