

# pH-Induced Conformational Changes in the Soluble Manganese-Stabilizing Protein of Photosystem II†

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**ABSTRACT:** In this paper, we analyzed the pH-induced changes in the conformational states of the manganese-stabilizing protein (MSP) of photosystem II. Distinct conformational states of MSP were identified using fluorescence spectra, far-UV circular dichroism, and pressure-induced unfolding at varying suspension pH values, and four different conformational states of MSP were clearly distinguished using the center of fluorescence spectra mass when suspension pH was altered from 2 to 12. MSP was completely unfolded at a suspension pH above 11 and partly unfolded below a pH of 3. Analysis of the center of fluorescence spectral mass showed that the MSP structure appears stably folded around pH 6 and 4. The conformational state of MSP at pH 4 seems more stable than that at pH 6. Studies of peak positions of tryptophan fluorescence and MSP-bound 1-anilinoanthracene-8-sulfonic acid fluorescence spectra supported this observation. A decrease in the suspension pH to 2 resulted in significant alterations in the MSP structure possibly because of protonation of unprotonated residues at lower pH, suggesting the existence of a large number of unprotonated amino acid residues at neutral pH possibly useful for proton transport in oxygen evolution. The acidic pH-induced conformational changes of MSP were reversible upon increase of pH to neutral pH; however, *N*-bromosuccinimide modification of tryptophan (Trp241) blocks the recovery of pH-induced conformational changes in MSP, implying that Trp241 is a key residue for the unfolded protein to form a functional structure. Thus, pH-induced structural changes of stable MSP (pH 6–4) may be utilized to analyze its functionality as a cofactor for oxygen evolution.

Photosystem II (PSII)<sup>1</sup> catalyzes the light-driven reduction of plastoquinone and oxidation of water to molecular oxygen (*I*). The major components of PSII required for its activity include chlorophyll *a* (P680), tyrosine residue (*Y<sub>Z</sub>*), pheophytin (Pheo), and primary (*Q<sub>A</sub>*) and secondary (*Q<sub>B</sub>*) quinone acceptors; all of them are located within a heterodimeric matrix consisting of polypeptides D1 and D2. Although the

essential pattern of photosynthetic water oxidation into molecular oxygen and protons has been established firmly, a detailed comprehensive picture of water oxidation is still far from a satisfying description. This is especially true for the manganese cluster of the water-oxidizing complex (WOC). Site-directed mutagenesis experiments have revealed that several amino acid residues of D1 and D2 are of functional and/or structural relevance in establishing a competent WOC. However, other polypeptides cannot be excluded for their potential to be essential constituents of WOC (2).

Among the proteins that function in PSII oxygen evolution, the extrinsic 33 kDa manganese-stabilizing protein (MSP) has special significance, because it stabilizes the manganese cluster under physiological conditions. The removal of this protein from PSII membranes results in the loss of two Mn<sup>2+</sup> ions and a significant decrease in oxygen evolution at a low chloride level (3, 4). Although the release of Mn is prevented by the addition of high concentrations of Cl<sup>–</sup> to a MSP-depleted PSII suspension, the oxygen evolution activity recovered is only 50% of that in intact PSII membranes (5). Spinach MSP is composed of 247 amino acid residues, including one tryptophan (Trp241), eight tyrosine, and two cysteine residues (Cys28 and Cys51 form a disulfide bridge). The crystal structure of oxygen-evolving PSII (including MSP) was recently resolved at 3.8 and 3.7 Å resolutions for

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<sup>1</sup> Abbreviations: ANS, 1-anilinoanthracene-8-sulfonic acid; CCD, charge-coupled device; CD, circular dichroism; Chl, chlorophyll; CSM, center of spectral mass; DMBQ, 2,6-dimethyl-*p*-benzoquinone; FTIR, Fourier transform infrared; MES, 4-morpholineethanesulfonic acid; MSP, manganese-stabilizing protein; NBS, *N*-bromosuccinimide; *P*<sub>1/2</sub>, experimental half-pressure of denaturation; PSII, photosystem II; Trp, tryptophan; UV, ultraviolet; WOC, water-oxidizing complex.

two species of thermophilic cyanobacterium (6, 7). The detailed structure of MSP, however, has not been elucidated yet.

The C terminus of MSP is critical for its binding to PSII and restoration of O<sub>2</sub> evolution (8, 9). The presence of a synthetic C-terminal peptide diminished the restoration effect of MSP on the oxygen-evolving activity of MSP-depleted PSII membranes (10). Cross-linking between Lys48 and Glu246 revealed that the N- and C-terminal domains are close together (11), and both are involved in MSP binding to PSII. Recent studies convincingly show that the Val148–Gly163 domain containing several key charged residues is useful for functional interaction between MSP and PSII (12). *N*-bromosuccinimide (NBS) modification of Trp241 leads to a large increase in the amount of random coils and loss of  $\alpha$ -helix and  $\beta$ -sheet structures of MSP, suggesting that this residue is essential for maintaining an active conformation and that the hydrophobic domain around this residue prevents nonspecific binding of the protein to PSII (13).

Another useful method to characterize MSP involves analysis of the isolated protein in solution. Circular dichroism (CD) and Fourier transform infrared (FTIR) studies on MSP in solution revealed a secondary structure characterized by high antiparallel  $\beta$ -sheet content and turns, together with a low fraction of  $\alpha$  helices (14). This was confirmed by information from PSII crystal structures (6, 7). However, various data on structural content are currently highly inconsistent. MSP is conformationally flexible in solution (15–17) and thus classified as a “natively unfolded” protein (16) or a “molten globule” (17). MSP exhibits unusual hydrodynamic mobility in solution. The molecular mass of the protein is 26.5 kDa, as predicted from its amino acid sequence, which was verified by matrix-assisted laser desorption ionization mass spectrometry (18, 19). The molecular mass derived from the mobility on SDS–PAGE was 33 kDa (20) and that derived from gel filtration columns was 37–40 kDa (8); these values are significantly larger than the molecular mass predicted. In addition, spinach MSP displays a specific thermostability (16) and an acidic pI value (5.2) (20). The study on the folding–unfolding characteristics of MSP with guanidine hydrochloride (Gdn-HCl) and high pressure disclosed that the protein has very small free energy ( $\Delta G_0$ ) for unfolding. MSP unfolds extremely easily following treatment with 2 M Gdn-HCl or under relatively low pressure (about 160–180 MPa) (21, 22). FTIR spectroscopy and cross-linking data showed that the secondary structure of MSP in solution varies over a wide range (15, 11). In view of these results, further studies on MSP are required to obtain a clearer picture.

Spinach MSP contains a single highly conserved tryptophan residue (Trp241), assumed to be embedded within a hydrophobic  $\beta$ -sheet environment near the C terminus (13, 23, 24). Trp fluorescence was successfully employed in studies on MSP unfolding (21, 22). Trp fluorescence of MSP is altered markedly when structural changes are induced via a reductive break of the conserved S–S bridge (21). Thus, fluorescence emission of Trp241 is a useful intrinsic indicator.

Isolated WOC exhibits pronounced pH-dependent structural changes (25, 26), which may be related to the protonation or deprotonation of relevant residues in MSP. Titration of the isolated MSP displayed an acid/base hys-

teresis in solution, which may be of physiological relevance in regulating the adaptation of WOC to different pH ranges within the lumen. Thus, pH-dependent modification of MSP in solution may be of value in determining the molecular mechanism of action of this protein (27). In this paper, pH-induced structural changes of isolated MSP were analyzed using fluorescence spectra, CD, and pressure-induced unfolding.

## MATERIALS AND METHODS

**Purification of MSP.** PSII membranes were isolated from market spinach leaves using the method of Berthold et al. (28), and treated with 1.5 M NaCl at 1.0 mg of chlorophyll (Chl) mL<sup>−1</sup> for 1 h at 4 °C under normal room light. After centrifugation at 40000g for 20 min, pellets were washed with 1 M NaCl. The suspension was immediately recentrifuged at 40000g for 20 min. Pellets were incubated in a solution of 0.4 M sucrose, 50 mM 4-morpholineethanesulfonic acid (MES)-NaOH (pH 6.2), 15 mM NaCl, 10 mM MgCl<sub>2</sub>, and 1 M CaCl<sub>2</sub> at 4 °C for 30 min in the dark. After another centrifugation at 40000g for 20 min, the supernatant was dialyzed overnight against 5 mM MES-NaOH (pH 6.2). Crude extracts were purified by column chromatography on a DEAE-Sephacrose CL-6B column, as described by Kuwabara and Murata (29). Purified protein was dialyzed against 10 mM phosphate buffer (pH 6.0). The MSP concentration was calculated from UV absorbance at 276 nm, according to the method of Eaton-Rye and Murata (9).

**Measurement of Fluorescence Spectra.** Fluorescence spectra were measured with an Aminco Bowman SLM 48000 fluorospectrometer (SLM Co.) in which the fluorescence detector was replaced by a charge-coupled device (CCD) detector (HAM 122 × 1024B). The sample holder was replaced with a homemade component to measure fluorescence under pressure from 0.1 to 300 MPa through thermostated pressure bombs. Spectral shifts in fluorescence were quantitated by specification of the center of spectral mass  $\langle \nu \rangle$ , a parameter defined by the equation:

$$\langle \nu \rangle = \frac{\int \nu F(\nu) d\nu}{\int F(\nu) d\nu} \quad (1)$$

where  $F(\nu)$  stands for the fluorescence emitted at wavenumber  $\nu$  and  $\int F(\nu) d\nu$  is the spectral integral. Excitation wavelengths were 295 nm for tryptophan and 390 nm for 1,8-ANS (1-anilinonaphthalene-8-sulfonic acid) as an extrinsic fluorescence probe. The degree of unfolding or degree of transition ( $\alpha$ ) is related to  $\langle \nu \rangle$  by the formula:

$$\alpha = [1 + Q(\langle \nu \rangle_p - \langle \nu \rangle_U) / (\langle \nu \rangle_N - \langle \nu \rangle_p)]^{-1} \quad (2)$$

where  $Q$  is the ratio of the quantum yields of the unfolded state over the native state and  $\langle \nu \rangle_p$ , the center of spectral mass at pressure  $p$ .  $\langle \nu \rangle_U$  and  $\langle \nu \rangle_N$  are the corresponding quantities for the unfolded and native states, respectively.

**Acid–Base Titration.** Titration was performed at 20 °C by adding very small aliquots of highly concentrated HCl or NaOH into a 10 mM phosphate buffer. A stable pH value was maintained for 5 min, after which the fluorescence spectra of MSP samples were immediately recorded. For details in pressure-induced unfolding, see caption of Figure 2.

**Measurement of CD Spectroscopy.** Before CD measurement, the MSP solution was filtered through a polyethersulfone membrane (0.2  $\mu\text{m}$ ) for purification. CD spectra were measured with a Jasco J-715 spectropolarimeter at 20  $^{\circ}\text{C}$ . The cell length was 1 mm. Data were collected every 0.1 nm with a 1 nm bandwidth and a 1 s time constant at a scan speed of 10 nm  $\text{min}^{-1}$ .

**Chemical Modification of MSP with NBS.** NBS modification was performed following the method of Yu et al. (13) at pH 2.5. Modification was stopped when no further decrease in absorption at 280 nm was observed with sequential addition of 1 mL of 10 mM NBS (Sigma).

**Reconstitution of MSP with PSII Membranes.** PSII membranes were suspended using  $\text{SCN}_{\text{low}}$  solution (0.4 M sucrose, 10 mM  $\text{CaCl}_2$ , 10 mM NaCl, and 50 mM MES-NaOH at pH 6.2) into 1.0 mg of Chl  $\text{mL}^{-1}$ . The suspension was treated with 2.6 M urea/0.2 M NaCl for 30 min at 4  $^{\circ}\text{C}$  in the dark. Pellets were resuspended in  $\text{SCN}_{\text{high}}$  solution ( $\text{SCN}_{\text{low}}$  solution containing 180 mM NaCl) after centrifugation at 40000g for 20 min.

Before reconstitution, NBS-modified MSP was dialyzed against 50 mM MES-NaOH at pH 6.2, for 3 h. Afterward, the solution was filtered through a polyethersulfone membrane (0.2  $\mu\text{m}$ ) for purification, and the protein concentration was determined. Absorption of NBS-modified MSP was corrected after taking into account the modification-caused decrease. For reconstitution, the concentrations of urea/NaCl-treated PSII membranes were adjusted to 0.1 mg of Chl  $\text{mL}^{-1}$ . MSP was added into the reaction medium to obtain the desired protein-to-PSII membrane ratio. The ratio of NBS-modified MSP to PSII was 20:1. For unmodified MSP, the ratio was 8:1. PSII membranes and MSP were incubated at 4  $^{\circ}\text{C}$  for 30 min in the dark and centrifuged at 40000g for 20 min. Pellets were washed twice with  $\text{SCN}_{\text{low}}$  solution to remove loosely bound MSP.

**Measurement of the Oxygen-Evolving Activity.** The oxygen-evolving activity of PSII membranes was determined to be 240  $\mu\text{mol}$  of  $\text{O}_2$  (mg of Chl) $^{-1}$   $\text{h}^{-1}$  with a Clark-type oxygen electrode in  $\text{SCN}_{\text{low}}$  solution at 25  $^{\circ}\text{C}$ . The Chl concentration in the reaction medium was 10  $\mu\text{g}$   $\text{mL}^{-1}$ . 2,6-Dimethyl-p-benzoquinone (DMBQ) (0.8 mM) was employed as the artificial electron acceptor.

## RESULTS

**Fluorescence Data Confirm the Existence of Different MSP Conformational States at Various pHs.** Tryptophan is an intrinsic fluorescence probe used to reflect changes in its immediate hydrophilic/hydrophobic microenvironment and the conformational states of relevant proteins. At neutral pH, Trp241 in soluble MSP is located in a hydrophobic region in a predicted  $\beta$ -sheet conformation (23, 24). MSP fluorescence spectra were measured by selecting 295 nm as the excitation wavelength, which excites tryptophan residues only and does not excite tyrosine residues in proteins. In total, we measured 30 fluorescence emission spectra. For a clear presentation, Figure 1A depicted 8 of these spectra. Figure 1B showed the changes of the fluorescence peak position of MSP upon changing pH.

At acidic pH (pH 2), the MSP spectrum showed a peak at 334 nm. When the suspension pH was increased, the peak displayed a blue shift with the maximum intensity appearing

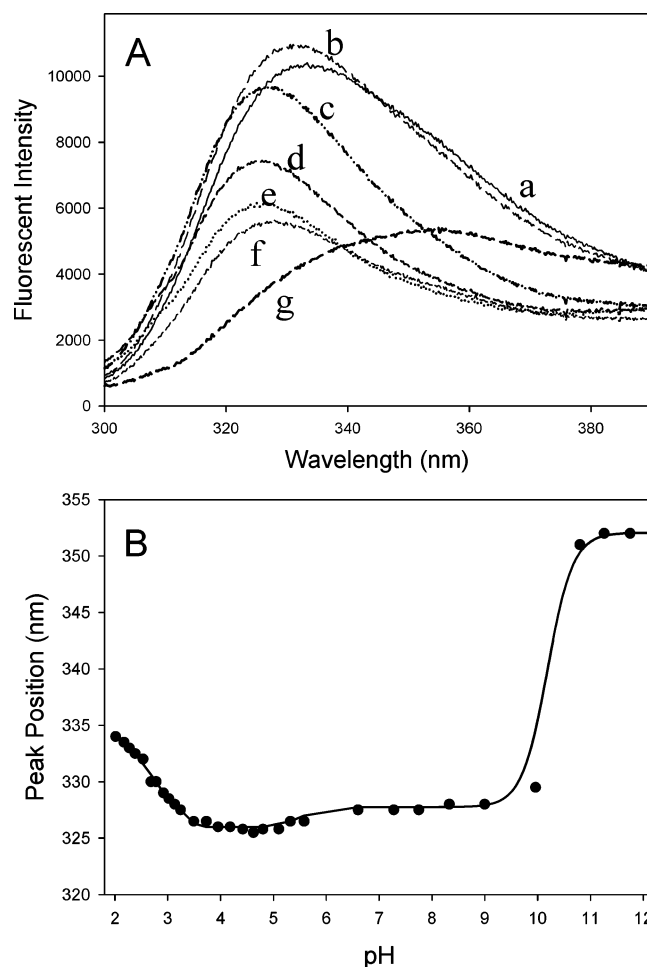


FIGURE 1: Intrinsic trp fluorescence emission spectra (A) and emission peak position (B) of MSP measured as a function of suspension pH from 2.0 to 11.8. Excitation wavelength = 295 nm. pH values: a, 2.0; b, 2.5; c, 3.5; d, 4.4; e, 5.1; f, 7.3; g, 11.8. Protein concentration = 0.3 mg/mL.  $T = 25^{\circ}\text{C}$ . For details, see the Materials and Methods.

at pH 2.5. At pH values higher than 2.5, the peak intensity started to decrease. Meanwhile, the blue shift continued until a position at 325 nm at around pH 4.4. Upon further increase in suspension pH, the wavelength of the peak began to show a red shift with no accompanying significant changes in intensity. At pH values higher than 11, no further spectral changes were found in either peak position (350 nm) or intensity. Because 350 nm is identical to the wavelength of the fluorescence peak of free tryptophan in a water solution, we propose that Trp241 of MSP is exposed to a polar environment at pHs higher than 11. The results suggest that MSP is extremely unfolded at pHs higher than 11. In contrast, the emission peak position of 334 nm at pH 2 indicates that Trp241 of MSP is located in a relatively hydrophobic environment. These results clearly indicate the existence of different conformational states of MSP at varying suspension pH values from 2 to 12.

**Pressure-Unfolding Evidence for the Existence of Different MSP Conformational States at Various pHs.** Using eq 2, the unfolding degree was evaluated from center of spectral mass (CSM) of trp fluorescence spectra. MSP-unfolding degree curves, representing pressure-induced unfolding at different suspension pH values, are shown in Figure 2. At pH 8.8, the unfolding curve displayed a sigmoidal shape. This



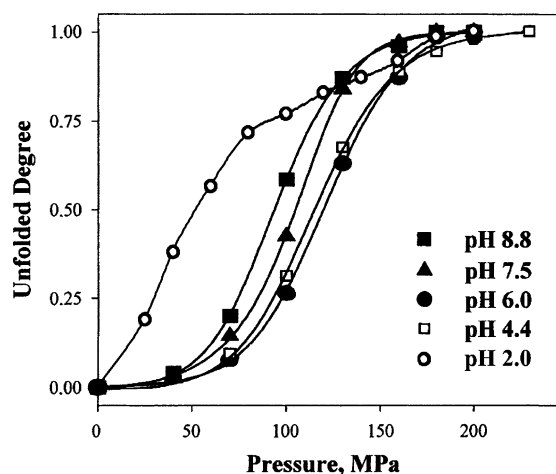


FIGURE 2: Pressure-induced unfolding curves of MSP at different suspension pH values. Data for pHs other than 2 are identical to ref. 22. The protein concentration was 0.1 mg/mL in medium containing 0.01 M HCl (pH 2), 0.05 M Mes-NaOH (pH 4.4 and 6.0), or 0.05 M Tris-HCl (pH 7.5 and 8.8).  $T = 25^\circ\text{C}$ . For details, see the Materials and Methods.

indicates that the pressure-induced unfolding is a two-state process, which took place as the pressure was raised from 40 to 140 MPa, with a  $P_{1/2}$  value (the pressure at which the degree of unfolding,  $\alpha$ , is 0.5) of 94 MPa. Upon sequential decrease of suspension pH from 8.8 to 7.5, 6.0, and 4.4, the  $P_{1/2}$  value changed from 94 to 105, 118, and 116 MPa, respectively. However, the sigmoidal character of unfolding degree curves remained unchanged. At pH 4.4, the two-state unfolding process took place as the pressure was raised from 50 to 150 MPa. From the above unfolding curves, conformational change between two distinguished states at each suspension pH value could be easily dissolved. However, when medium pH was decreased from 4.4 to 2.0, the characteristics of the unfolding curve altered dramatically. At pH 2, the unfolding curve was not sigmoidal, implying that the two-state unfolding process does not exist with increasing pressure. This means that the initial conformational state of MSP at pH 2 is distinct from that at other pH values stated above. We suggest that MSP is in an unfolded state at pH 2.

The largest  $P_{1/2}$ , 116 or 118 MPa, was observed at suspension pH values of 4.4 or 6.0. The data imply that it is easier to unfold MSP at suspension pH values higher than 6.0 or lower than 4.4.

**CD Evidence for the Existence of Different MSP Conformational States at Various pHs.** Monitoring of the CD spectrum in the far-UV region (190–250 nm) is another useful tool to determine secondary structures of proteins. Figure 3 depicted the different far-UV CD spectra of MSP when suspension pH was increased from 2 to 12. At neutral pH (6), the CD spectrum displayed a positive peak at 197 nm and a flattened negative peak at 224 nm. When suspension pH was decreased from 6 to 4, no major changes were observed in the positive peak. The negative peak around 224 nm became slightly deeper and broader, indicating that the decrease in pH from 6 to 4 does not significantly affect the basic characteristics of the CD spectrum. Thus, the major secondary structure of MSP remains unaffected at pH 4. Moreover, our data are consistent with the results that the secondary structure elements of MSP remained constant upon

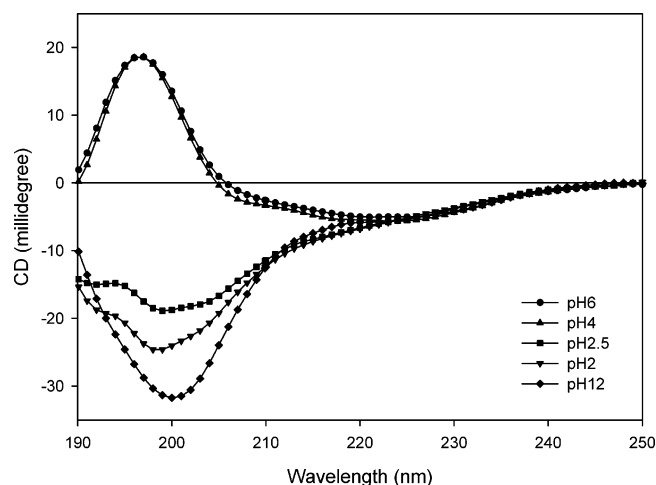


FIGURE 3: Far-UV CD spectra of MSP at different suspension pH values. Protein concentration = 15  $\mu\text{M}$ .  $T = 25^\circ\text{C}$ . For details, see the Materials and Methods.

decrease of pH from 6.8 to 3.8 (27). However, notably, the decrease of pH from 6 to 4 induced a trivial spectral change around 220 nm, indicating a slight state transition, although both are ordered states (see the Discussion below). After further acidification (decreasing pH from 4 to 2), the CD spectra from 190 to 220 nm changed significantly. At pH 2, the CD spectrum of MSP contained a negative, instead of a positive, peak near 200 nm. This negative peak is a typical structural feature of proteins, often attributable to random coils. This significant change indicates that the percentage of secondary structure of MSP was dramatically changed at pH 2, presumably because of protonation of unprotonated residues. At pH 12, on the other hand, the ellipticity at 200 nm is much lower than that at pH 2. This finding additionally suggests that the alkaline suspension medium at pH 12 induced significant changes in the MSP conformation with an increase in the amount of random coils, possibly caused by over deprotonation of related amino acid residues. Thus, both acidic and alkaline pH values altered the MSP conformation from a relatively ordered state (at neutral pH) to disordered states.

**Identification of the Four Different Conformational States of MSP at a pH Range from 2 to 12.** For an accurate measurement of spectral shifts, centers of spectral mass were calculated from data in Figure 1A (Figure 4). Generally, a higher CSM value indicates a shorter wavelength of maximum emission. Two plateaus were observed in the CSM curve. One is from pH 3.5 to 4.8, and the other is from pH 6.0 to 9.5, indicating that the MSP structure is relatively stable within these pH regions. Furthermore, the different CSM values indicate that the conformational states within these pH ranges are slightly different, consistent with previous CD results at pH 6 and 4. At an approximate pH of 11.3 or higher, no further change in the CSM value was observed. The CSM value increased from pH 2.0 to 3.5 but decreased from pH 4.8 to 6.0. At a pH value over 9.5, a significant decrease in the CSM value was observed. In summary, four structurally different states of MSP could be identified, namely, two stable states at pH 3.5–4.8 and pH 6.0–9.5, as well as two unstable states at pH 2.0–3.5 and pH 9.5–12, respectively.

To obtain more lines of evidence supporting the observation with trp fluorescence data, 1,8-ANS, a hydrophobic

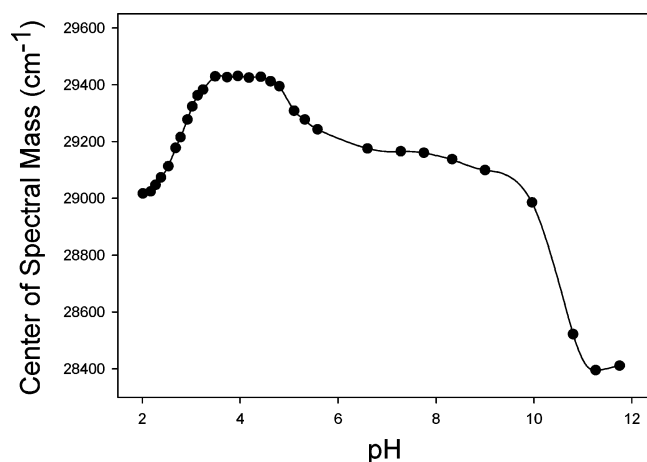


FIGURE 4: The CSM calculated from fluorescence spectra of MSP in Figure 1A. Protein concentration = 0.3 mg/mL.  $T = 25^{\circ}\text{C}$ . For details, see the Materials and Methods.

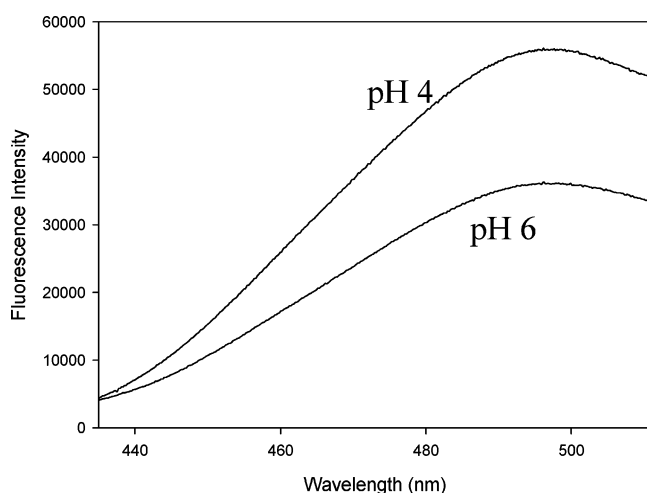


FIGURE 5: Fluorescence spectra of the ANS-binding MPS at different suspension pH values. Excitation wavelength = 390 nm (excitation only ANS). Protein concentration = 0.3 mg/mL. ANS concentration =  $1\ \mu\text{M}$ .  $T = 25^{\circ}\text{C}$ . For details, see the Materials and Methods.

fluorescent probe, was used. The emission spectrum of 1,8-ANS depends on the accessibility of hydrophobic domains within the protein to this water-soluble fluorophore (30). Figure 5 depicts the dependence of fluorescence spectra of ANS bound to MSP on different suspension pH values. Free ANS in a water solution at pH 6 or 4 has little fluorescence emission, which can be ignored during analysis (data not shown). At pHs 6 and 4, the MSP-bound ANS fluorescence spectra contained similar peaks at 495 nm but with different intensities. Peak intensity increased when suspension pH was decreased from 6 to 4. It indicates that at pH 4, the microenvironment of ANS is relatively hydrophobic as compared to pH 6. Two different, pH-dependent stable states were distinguished. ANS fluorescence study, together with the results of trp fluorescence peak position (Figure 1B) and CSM of trp fluorescence spectra (Figure 4), supported the conclusion that the conformational state of MSP at pH 4 is more stable than that at pH 6.

**NBS Modification of Trp241 Blocks the pH-Induced Conformational Recovery of MSP.** NBS modification of Trp241 is a useful tool for the study of soluble MSP. NBS modification was performed following the method of Yu et

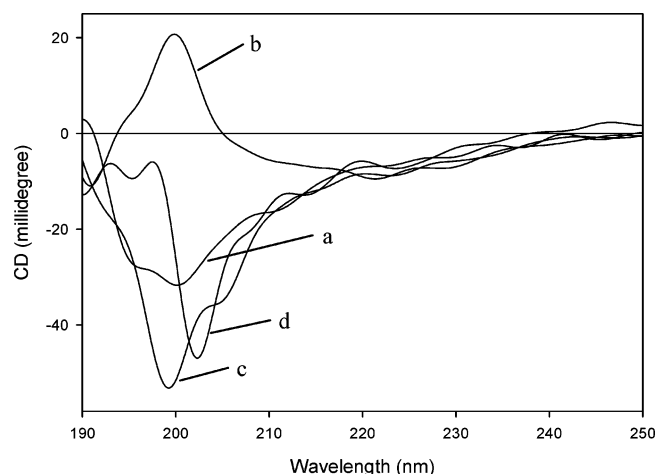


FIGURE 6: CD spectra of MSP with or without NBS modification. (a) unmodified MSP at pH 2.5; (b) unmodified MSP at pH 6.0; (c) NBS-modified MSP at pH 2.5; (d) NBS-modified MSP at pH 6.0. Protein concentration =  $10\ \mu\text{M}$ . For details, see the Materials and Methods.

al. (13). The ellipticity at 200 nm in CD spectra of NBS-modified and control MSP was altered from positive at neutral pH to negative at pH 2.5 (Figure 6). The ellipticity at 200 nm of NBS-modified MSP was lower than that of the nonmodified protein. This finding indicates that acidification of the suspension medium and not NBS modification of Trp241 induces significant conformational changes in MSP. NBS modification of Trp241 in MSP only facilitates an increase in the amount of random coil structure. After readjusting the suspension pH from 2.5 to 6, the CD spectrum of nonmodified MSP was similar to that of the original, indicating that pH-induced structural changes of MSP are reversible (Figure 6). Conformational changes of MSP are possibly caused by the deprotonation of interior residues of MSP at neutral pH and protonation at low pH. The CD spectrum of NBS-modified MSP displayed slight differences after suspension pH was readjusted to 6 but still contained a large amount of random coil. Reconstitution of NBS-modified MSP with MSP-depleted PSII membranes revealed that the NBS-modified protein lost the ability to specifically bind to PSII membranes. The oxygen-evolving activity of reconstituted PSII membranes with unmodified MSP was determined to be  $116\ \mu\text{mol of O}_2\ (\text{mg of Chl})^{-1}\ \text{h}^{-1}$  (49% of that in the intact PSII membranes). However, the oxygen-evolving activity of PSII membranes reconstituted with NBS-modified MSP was determined to be  $49\ \mu\text{mol of O}_2\ (\text{mg of Chl})^{-1}\ \text{h}^{-1}$ , which was similar to that of MSP-depleted PSII membranes ( $45\ \mu\text{mol of O}_2\ (\text{mg of Chl})^{-1}\ \text{h}^{-1}$ ). This is identical with the previous result of Yu et al. (13). This finding indicates that NBS-modified MSP had lost the physiological ability to restore oxygen-evolving activity upon reconstitution with the PSII membranes.

## DISCUSSION

Using the techniques of biophysics such as fluorescence measurements (Figures 1 and 5), pressure-induced conformational changes (Figure 2) and CD spectra (Figure 3), different pH-related structural states of isolated MSP were distinguished very clearly. Furthermore, using the fluorescence CSM method, four structural states were obtained for soluble MSP as the suspension pH was increased from 2.0

to 12 (Figure 4). When the suspension pH was increased from 2.0 to 3.5, the conformation of isolated MSP was altered from an unfolded to an ordered state. Upon further increase of suspension pH from 9.5 to 12, soluble MSP changed conformation from an ordered to another unfolded state. Data from Figures 1, 3, and 4 collectively indicate that the conformational state of soluble MSP at pH 12 is significantly more unfolded than that at acidic pH (e.g., pH 2). It has been reported that the oxygen evolution capability of thylakoids strongly depends on pH with a flat maximum between  $6.0 < \text{pH} < 8.0$  and steep declines in the acidic and alkaline range beyond the plateau region (25). It is possible that pH-induced conformational change in soluble MSP may indeed occur within the lumen.

Most important of all, two stable conformational states of MSP were identified, one at pH 3.5–4.8 and the other one at pH 6.0–9.5, with a transient pH from 4.8 to 6.0 between these two states. These two states differ slightly with each other as evidenced from their slightly different CSM values of Trp fluorescence. The existence of these two slightly different states is verified by fluorescence of 1,8-ANS and trp fluorescence peak position at pH 4.0 and 6.0, respectively. It is also supported by pressure-induced unfolding experiments from pH 4.4 to 8.8. However, the CD spectra of MSP are very similar between pH 4.0 and 6.0, implying that the major secondary components of MSP are not much different between these two states. Two stable conformational states of MSP might imply the existence of one highly protonated and the other highly deprotonated state in a complete oxygen evolution process. Detailed study on the transition between these two stable conformational states will be valuable in realizing the mechanism of oxygen evolution.

In photosynthetic oxygen evolution, four protons are generated for every oxygen molecule released from two water molecules oxidized. Protons release to the outside through hydrogen-bonded chains, most likely through amino acids in extrinsic proteins (33, 23, or 17 kDa), as a result of charge compensation in the primary photochemical reactions. To the best of our knowledge, Shutova et al. (27) were the first to propose a  $\text{H}^+$  channel through MSP. However, solid experimental evidence is still required to confirm this assumption. Proton release may occur from residues at the protein–water interface of MSP relatively distant from the catalytic site. Because of diversity, it is difficult to deduce the origins of protons detected during the oxygenic circle. We showed that decreasing the suspension pH from 4 to 2 altered the MSP conformation from an ordered to an unfolded state. This finding implies that, in the interior of MSP at neutral pH (and even pH 4), a number of protonatable residues possibly form chains of proton transport or provide ligands for the Mn cluster (or other cofactors). Recently, it was suggested that Glu residues in MSP could be possible candidates for the proton-transfer reaction (31). With the increase in the proton amount in the suspension medium at pH 2, the MSP structure may be rearranged into an unfolded state because of protonation of almost all protonatable residues. It is also interesting that over-deprotonation (at pH 12) of protonatable residues within MSP induces another unstable, unordered state.

The finding that the largest CSM value appears at pH 4 but not 6 (Figure 4) is consistent with ANS fluorescence data (Figure 5). The CSM results demonstrate that the

conformational state of soluble MSP around pH 4 is significantly more ordered than that around pH 6, indicating that protonation of protonatable residues in MSP, to some extent, induces structural rearrangement (most likely of the microenvironment) to a favorable state. The two stable states of isolated MSP observed at different suspension pH ranges (pH 3.5–4.8 and 6.0–9.5) may provide a novel insight into the molecular mechanism of photosynthetic oxygen evolution. Although the pH influence on soluble MSP may be different from that on MSP *in vivo* in PSII membranes, the two mutually exchangeable stable conformations of isolated MSP at pH 4 and 6 may be of relevance for oxygen evolution. First, under physiological conditions, WOC, closely related to proton accumulation or release, operates in a wide range of lumen pH. The existence of two apparently stable states of soluble MSP (from pH 3.5 to 9.5) is in agreement with the physiological requirements of green plants. Second, photosynthetic oxygen evolution, a dynamic self-regulatory process, may also modify the local proton concentration inside WOC significantly. The pH of the thylakoid lumen decreases rapidly upon illumination (down to values below pH 5.0 under saturating light) (32, 33), and light-induced  $\Delta\text{pH}$  across the thylakoid membrane disappears in the dark. Accumulated changes in local proton concentrations in the interior of membrane-bound MSP are additionally displayed in suspension pH. Stable states of MSP conformation are indicators of the different cumulative local proton amounts. Therefore, it is feasible to distinguish two pH-related stable states of MSP corresponding to the different redox states of WOC.

Probably, the finding that the soluble MSP conformation is most stable at suspension pH of 3.5–4.8 but not 6.0–9.5 is consistent with the previous observation that the oxygen evolution activity in PSII-enriched membranes is maximal when suspension pH is lower (pH 5.5–6.5) but not neutral (34), although corresponding pH ranges in the two cases are not identical. The fact that the water oxidase is inferred to undergo structural changes at pH values of 5.0–5.5 and 6.5–7.0 (25) also would be very helpful to realize that pH-induced conformational change in soluble MSP may indeed occur in the water oxidase. The relevance of pH-induced conformational changes of isolated MSP for analyzing structure–function relationships in WOC that are modulated by membrane-bound MSP in response to pH changes in the thylakoid lumen remains to be further clarified.

The far-UV CD spectrum of control MSP at pH 2.5 is similar to that of the protein modified with NBS (Figure 6). Thus, the acidification of the suspension medium but not NBS modification of Trp241 induces significant changes in the MSP conformation. In the absence of NBS, the pH-induced change of the MSP structure is reversible. The original conformation was recovered when the suspension pH was readjusted to 6. However, after NBS modification, the MSP conformation was not reversible as the suspension pH was increased to 6. We conclude that NBS modification of Trp241 prevents the deprotonation of related amino acids in MSP to form the original protein structure upon increase of the suspension pH from 2.5 to neutral. A previous study showed that NBS-modified MSP cannot specifically reconstitute with MSP-depleted PSII membranes (13) and NBS-modified MSP cannot restore the oxygen-evolving activity of reconstituted PSII membranes, in contrast to the unmodi-



fied protein. Thus, Trp241 may be a key residue for the sequential deprotonation of correlated amino acids that induces a functional conformation of MSP.

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