# Structural and Functional Differentiation of Three Groups of Tyrosine Residues by Acetylation of *N*-Acetylimidazole in Manganese Stabilizing Protein†

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ABSTRACT: To study its contribution to the assembly of the green plant manganese stabilizing protein (MSP) into photosystem II (PSII), tyrosine residues were specifically acetylated using *N*-acetylimidazole (NAI). In soluble MSP, three groups of Tyr residues could be differentiated by NAI acetylation: approximately 5 (actually ∼5.2) Tyr residues could be easily acetylated (superficial), 1−2 Tyr residues could be acetylated when the NAI concentration was sufficiently high (superficially buried), and 1−2 Tyr residues could only be acetylated in the presence of the denaturant, urea (deeply buried). Acetylation of the 5.2 Tyr residues did not affect the reconstitution or oxygen-evolving activities of the MSP, and far-UV circular dichroism (CD) analysis showed that the altered MSP retained most of its native secondary structure. These results suggested that the 5.2 Tyr residues are not absolutely essential to the function of MSP. However, further modification of the 1−2 superficially buried Tyr residues (for a total acetylation of ∼6.4 Tyr residues) completely abrogated the MSP rebinding and oxygen evolution activities. Finally, at least one tyrosine residue was inaccessible to NAI until MSP was completely unfolded by 8 M urea. Deacetylation of MSP with 6.4 or 8 acetylated Tyr residues with hydroxylamine restored most of the rebinding and oxygen-evolving activities. A prominent red shift in fluorescence spectra of MSP (excited at 280 or 295 nm) was observed after modification of 6.4 Tyr residues, and a further shift could be found after all 8 Tyr residues were modified, indicating a great loss of native secondary structure. Far-UV CD revealed that MSP was mostly unfolded when 6.4 Tyr residues were modified and completely unfolded when all 8 Tyr residues were modified. Fluorescence and far-UV CD studies revealed that loss of MSP rebinding to PSII membranes following NAI modification correlated well with conformational changes in MSP. Together, these results indicate that different tyrosine residues have different contributions to the binding and assembly of MSP into PSII.

In green plants, photosystem II (PSII)[1] catalyzes the light-driven reduction of plastoquinone and the oxidation of water to molecular oxygen (*1*). All major components of PSII required for its activity, including chlorophyll *a* (P680), tyrosine ($Y_Z$), pheophytin (Pheo), and the primary ($Q_A$) and secondary ($Q_B$) quinone acceptors are located within a heterodimeric matrix consisting of polypeptides D1 and D2. Although the essential pathway for photosynthetic water oxidation into molecular oxygen and protons has been established, a detailed comprehensive picture of water oxidation has not been elucidated to date. In particular, the manganese cluster of the water-oxidizing complex (WOC) is not well understood. Site-directed mutagenesis experiments have revealed that several amino acid residues of D1 and D2 are functionally and/or structurally relevant for establishing a competent WOC. However, other extrinsic polypeptides cannot be excluded as being essential constituents of the WOC (*2*).

Among the proteins that function in PSII oxygen evolution, the extrinsic 33 kDa manganese stabilizing protein (MSP) is particularly important, as it stabilizes the manganese cluster under physiological conditions. Removal of MSP from PSII membranes results in the loss of two $Mn^{2+}$ ions and significant decreases in oxygen evolution at low chloride levels (*3*, *4*). Although this release of Mn can be prevented by the addition of high concentrations of $Cl^-$ to MSP-depleted PSII suspensions, the recovered oxygen evolution activity is only 50% of that seen in intact PSII membranes (*5*). CD and FT-IR spectra characterized soluble MSP as having a high content of β-sheets and turns and a low content of α-helices (*6*). On the basis of its specific thermostability, acidic isoelectric point, and anomalous electrophoretic

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[1] Abbreviations: CD, circular dichroism; Chl, chlorophyll; DMBQ, 2,6-dimethyl-*p*-benzoquinone; FTIR, Fourier tranform infrared; MES, 4-morpholineethanesulfonic acid; MSP, manganese stabilizing protein; NAI, *N*-acetylimidazole; PSII, photosystem II; WOC, water-oxidizing complex.

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mobility, MSP was classified as a "natively unfolded" (*7*) protein or a "molten globule" (*8*). Soluble MSP unfolds extremely easily in the presence of 2 M Gdn-HCl or under relatively low pressure (about 160−180 MPa) (*9*, *10*). The crystal structure of PSII and studies of pH-induced structural changes in MSP suggested that MSP might provide a pathway for transporting protons from the catalyzing site of oxygen evolution to the lumen (*11*−*13*). A recently determined 3D X-ray structure of oxygen-evolving PSII of the cyanobacterium *Thermosynechococcus elongates* at 3.5 Å resolution provided further structural information about MSP. It is proposed that MSP, located on the luminal surface within PSII, forms an eight-stranded $\beta$-barrel. The whole protein takes on an elongated shape with two major domains (*11*). On the basis of this, homologous models for eukaryotic MSP were built by using bioinformatics analysis. Five highly conserved regions were identified as the CC bridge and KL, GGER, DPKGR, and GEhhG regions. Among these, the CC bridge and GEhhG region seem to play roles in maintaining the functional structure of MSP while the others seem to be critical for the binding of MSP to PSII (*14*).

The C-terminus of MSP is known to be critical for the binding of MSP to PSII and the restoration of $O_2$ evolution (*15*−*17*). Spinach MSP is composed of 247 amino acid residues including one tryptophan residue (Trp241), two Cys residues (Cys28 and Cys51, forming a disulfide bridge), and eight Tyr residues (*18*). Trp241 is buried in a strong hydrophobic $\beta$-sheet region near the C-terminus (*19*−*21*) and is suggested to be a key residue through which unfolded MSP forms a functional structure (*19*). Cross-linking between Lys48 and Glu246 revealed that the N- and C-terminal domains are close together (*22*) and that both terminals are involved in the binding of MSP to PSII. Recent studies have demonstrated that the N-terminal domain ($^{15}$T−$^{18}$E) is required for binding of at least one copy of MSP to PSII in cyanobacteria, algae, and higher plants (*23*). The eight Tyr residues (Tyr8, Tyr16, Tyr45, Tyr83, Tyr110, Tyr150, Tyr167, and Tyr242) are distributed over the entire sequence of spinach MSP and are presumed to be primarily embedded within a hydrophobic microenvironment (*20*).

Previously, tryptophan fluorescence in MSP was used to study structural changes in the microenvironment of Trp241 (*9*, *10*). Furthermore, MSP displays a rare protein fluorescence under conditions promoting a Tyr residue emission spectrum, suggesting that tyrosine fluorescence could be used to provide information about Tyr residues in secondary structure of MSP (*20*, *21*).

Chemical modification of proteins has been used to study the localization of individual amino acids, their participation in the maintenance of native conformation, their stabilization and conversion to intermediate states. More specifically, chemical modification of specific amino acids can be used to clarify structure−function relationships of specific residues in a given protein. *N*-Acetylimidazole (NAI), first introduced by Riordan et al. (*24*, *25*), is a mild and selective protein-acetylating reagent that preferentially acetylates exposed Tyr residues in neutral pH but can also react with other amino acids such as serine and threonine (*26*). In this study, using a technical combination of chemical modification, fluorescence, and far-UV circular dichroism, we sought to differentiate the various Tyr residues within MSP and determine which are crucial for the function and secondary structure of MSP.

## MATERIALS AND METHODS

*Purification of MSP.* Spinach was purchased from a local market in Shanghai. PSII membranes (1.0 mg of Chl mL$^{-1}$) were isolated from the spinach leaves following the method of Berthold et al. (*27*) and then treated with 1.5 M NaCl for 1 h at 4 °C under normal room light. After centrifugation at 40000*g* for 20 min, pellets were resuspended in SMN medium [0.4 M sucrose, 50 mM MES−NaOH (pH 6.2), 15 mM NaCl, 10 mM $MgCl_2$] and mixed with the same volume of 2 M NaCl. This suspension was immediately centrifuged at 40000*g* for 20 min, and the pellets were further incubated in a solution of 0.4 M sucrose, 50 mM MES−NaOH (pH 6.2), 15 mM NaCl, 10 mM $MgCl_2$, and 1 M $CaCl_2$ at 4 °C for 30 min in the dark. The samples were subjected to a further centrifugation at 40000*g* for 20 min, and the supernatant was dialyzed overnight against 5 mM MES−NaOH (pH 6.2). The resulting crude extracts were purified by column chromatography on a DEAE-Sepharose CL-6B column, as described by Kuwabara and Murata (*28*), and the purified proteins were dialyzed against 10 mM phosphate buffer (pH 6.0) before use. The MSP concentration was calculated from UV absorbance at 276 nm, according to the method of Eaton-Rye and Murata (*16*).

*Chemical Modification of MSP Tyr Residues with NAI.* NAI stored under vacuum in a desiccator was freshly prepared immediately before use. NAI modification of MSP (40 $\mu$M) was carried out in 50 mM phosphate buffer (pH 7.5) at 37 °C in the presence of various concentrations of NAI (0.1−100 mM) for 2 h. For acetylation of denatured MSP, samples were incubated with 8 M urea for 1 h before 10 mM NAI was added. The number of Tyr modified residues was determined by the method of Riordan and Vallee (*26*). Therefore, different fractions of tyrosine residues can be clearly figured out using this method.

*Deacetylation by Hydroxylamine.* For deacetylation, hydroxylamine was added to NAI-modified MSP in phosphate buffer, pH 7.5 (hydroxylamine final concentration is 1 M). The mixture was incubated at room temperature for 1 h and then dialyzed against 50 mM MES−NaOH (pH 6.5) for 5 h for reconstitution of the deacetylated protein with PSII membranes.

*Measurement of the Fluorescence Spectra.* The fluorescence emission spectra of MSP were measured with a Hitachi F4010 fluorescence spectrophotometer. The concentration of MSP was 20 $\mu$M, and the excitation wavelength was set at 280 nm to excite both tryptophan and tyrosine or at 295 nm to excite tryptophan alone. The native or NAI/urea-modified MSP samples were dialyzed against 10 mM phosphate buffer (pH 6.5) for 5 h for renaturation prior to measurements.

*Circular Dichroism (CD) Spectroscopy of MSP.* For CD spectroscopy, the native or NAI/urea-modified MSP samples were transferred first to 10 mM phosphate buffer (pH 6.5) by extensive dialysis. After the MSP suspension had been filtered through a polyethersulfone membrane (0.2 $\mu$m) for purification, CD spectra were measured by a Jasco J-715 spectropolarimeter at 25 °C. The concentration of MSP was adjusted to 10 $\mu$M before each measurement. The cell length was 1 mm. Data were collected every 0.1 nm with 1 nm

bandwidth and 1 s time constant, at a scan speed of 10 nm $min^{-1}$.

*Reconstitution of MSP with PSII Membranes.* PSII membranes were suspended in $SCN_{low}$ solution (0.4 M sucrose, 10 mM $CaCl_2$, 10 mM NaCl, and 50 mM MES−NaOH, pH 6.5) to reach 1.0 mg of Chl $mL^{-1}$, and the suspensions were treated with 2.6 M urea/0.2 M NaCl for 30 min at 4 °C in the dark. After centrifugation at 40000*g* for 20 min, pellets were resuspended in $SCN_{high}$ solution ($SCN_{low}$ solution containing 180 mM NaCl).

Prior to reconstitution, various MSPs were dialyzed against 50 mM MES−NaOH (pH 6.5) for 3 h. Afterward, the solution was filtered through a polyethersulfone membrane (0.2 $\mu$m) for purification. For reconstitution, the concentrations of urea/NaCl-washed PSII membranes were adjusted to 0.1 mg of Chl $mL^{-1}$, and MSP was added into the reaction medium to obtain an 8:1 protein-to-PSII membrane ratio. The mixtures were incubated at 4 °C for 30 min in the dark and centrifuged at 40000*g* for 20 min, and the pellets were washed twice with $SCN_{low}$ solution for removal of loosely bound MSP.

*Measurement of the Oxygen-Evolving Activities.* The oxygen-evolving activity of PSII membranes was measured with a Clark-type oxygen electrode in $SCN_{low}$ solution at 25 °C. The chlorophyll concentration in the reaction medium was 10 $\mu$g $mL^{-1}$, and 0.8 mM 2,6-dimethyl-*p*-benzoquinone (DMBQ) was employed as the artificial electron acceptor.

*SDS−PAGE Analysis of Protein Content.* Protein content was analyzed with sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using a Laemmli system (*29*) of a slab gel containing a 5% stacking gel and an 11.75% resolving gel, both containing 6 mol/L urea. The resultant gels were stained with Coomassie Brilliant blue R-250, and densitograms were obtained with a Digital Imaging System (IS-1000). The relative amounts of MSP were determined by integrating the peak areas, with the amount of native MSP reconstituted with urea/NaCl-washed PSII set as 100%.

## RESULTS AND DISCUSSION

The acetylation degree of the Tyr residues in MSP was closely dependent upon the NAI concentration in the suspension medium (Figure 1). The acetylation curve of MSP displayed a sigmoidal shape with two plateaus where ~5.2 and 6.4 Tyr residues were acetylated, respectively. Approximately 5.2 MSP Tyr residues were modified by 10 mM NAI, corresponding to a 250-fold molar excess. It has to be indicated that 5.2 is an average value, implying that approximately five-eighth of Tyr residues in MSP could be modified.

For reconstitution, NAI-modified MSP was dialyzed against 50 mM MES−NaOH (pH 6.5) and then incubated with urea/NaCl-washed PSII membranes at 4 °C for 30 min in the dark. Loosely bound MSP was removed by washing twice with $SCN_{low}$ solution, and bound membranes were analyzed by SDS−PAGE (Figure 2). The gels contained normal and PSII membranes without extrinsic polypeptides (lacking the 33, 23, and 17 kDa peptides), respectively (Figure 2, lanes 1 and 2), PSII membranes rebound with unmodified MSP with an MSP-to-PSII ratio of 8:1 (mol/mol) in the reconstitution system (Figure 2, lane 3), and NAI-modified MSP treated with NAI from 0.1 to 10 mM (lanes 4−8 with an identical MSP-to-PSII ratio). The latter was able to rebind to the extrinsic polypeptide-depleted PSII membrane as effectively as the unmodified protein. After the 0.1−10 mM NAI-modified MSP was rebound with urea/NaCl-treated PSII membranes, the oxygen-evolving activity was restored to over 89% of the control value (Table 1). These data indicate that the ~5.2 easily acetylated Tyr residues in MSP are not crucial for its binding to PSII or its oxygen-evolving activity.

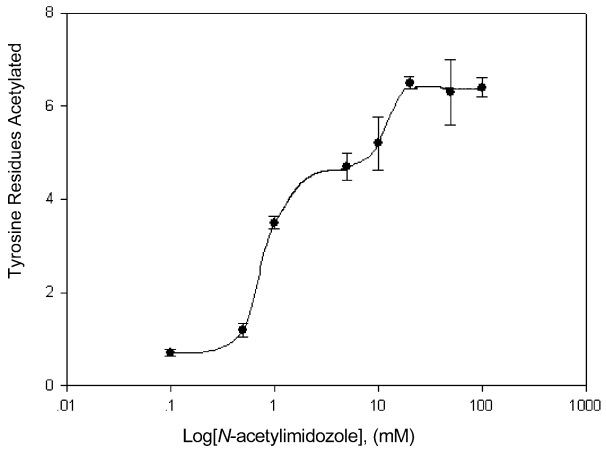



FIGURE 1: Acetylation of MSP with *N*-acetylimidazole (NAI). NAI modification of tyrosine in MSP (40 $\mu$M) was carried out in 50 mM sodium phosphate buffer (pH 7.5) at 37 °C for 1 h in the presence of NAI (0.1−100 mM). The amount of tyrosine residues modified was determined following the method of Riordan and Vallee (*26*). The results represent the mean ± SE of three experiments assayed in duplicate.

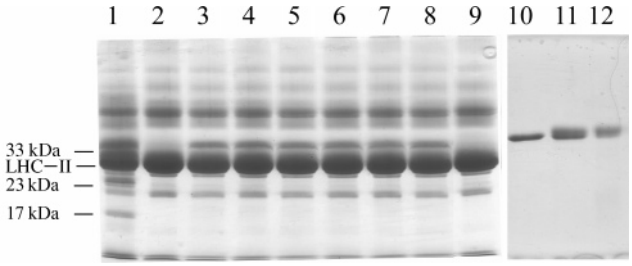



FIGURE 2: Reconstitution of NAI-modified MSP with 1 M urea/NaCl-washed PSII membranes. Lanes: 1, control PSII membranes; 2, 1 M urea/NaCl-washed PSII membranes; 10, native MSP; 11, MSP modified by 10 mM NAI; 12, MSP modified by 20 mM NAI; 3, PSII membranes reconstituted with native MSP; 4−10, PSII membranes reconstituted with MSP modified by NAI at concentrations of 0.1 (lane 4), 0.5 (lane 5), 1 (lane 6), 5 (lane 7), 10 (lane 8), and 20 (lane 9) mM, respectively. The noted 17, 23, and 33 kDa polypeptides are three extrinsic polypeptides involved in oxygen evolution; LHC-II = light harvesting complex II.

Fluorescence spectroscopy is a powerful tool for studying protein structure and function, as illumination at 280 nm excites both tryptophan and tyrosine residues in soluble proteins. The fluorescence spectrum of native MSP showed a maximum emission wavelength at 310 nm when excited at 280 nm (Figures 3a and 4A), which is consistent with a previous report (*19*). Ruan et al. (*21*) showed that the fluorescence emission of MSP excited at 280 nm is mainly dominated by tyrosine rather than tryptophan. As clearly shown in Figure 3, as the NAI concentrations increased from 0.1 to 10 mM, the emission intensity of the fluorescence peak progressively decreased with no change in the peak position, indicating that as the concentration of NAI increased

Table 1: Activity of Oxygen Evolution, Measured after Reconstitution of the Urea/NaCl-Washed PSII Membranes with Various MSPs

| sample | bound MSP (%) | oxygen evolving activity: $\mu$mol of $O_2$ (mg of Chl)$^{-1}$ h$^{-1}$ | oxygen evolving activity: % |
|---|---|---|---|
| (A) urea/NaCl-washed PSII | 0 | 35 | |
| (B) native MSP reconstituted with sample A | 100 | 136 | 100 |
| (C) renatured MSP reconstituted with sample A | 100 | 127 | 93.4 |
| (D) MSP (with 5.2 Tyr modified) reconstituted with sample A | 98 | 121 | 89 |
| (E) MSP (with 6.4 Tyr modified) reconstituted with sample A | 0 | 41 | 30.1 |
| (F) MSP (with 8 Tyr modified) reconstituted with sample A | 0 | 32 | 23.5 |
| (G) MSP, with 6.4 Tyr modified, reconstituted with sample A after deacetylation | 93 | 105 | 77.2 |
| (H) MSP, with 8 Tyr modified, reconstituted with sample A after deacetylation | 93 | 96 | 70.6 |

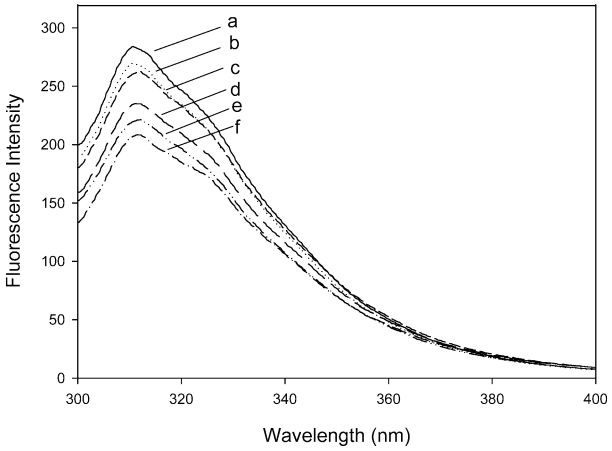



FIGURE 3: Fluorescence spectra of NAI-modified MSP. (a) Native MSP. (b−f) Native MSP incubated with 0.1, 0.5, 1, 5, and 10 mM NAI, respectively. For readings, MSP (20 $\mu$M) in 10 mM phosphate (pH 6.5) buffer was excited at 280 nm (25 °C), as described in Materials and Methods.

to 10 mM, the titratable Tyr residues were sequentially acetylated.

The intrinsic fluorescence of the tryptophan residues in a protein is extremely sensitive to the immediate hydrophilic/hydrophobic microenvironment; this can be used as a probe to reflect changes in the protein structure and conformation (*30*). The fluorescence spectrum of native MSP showed a maximum emission wavelength at 327 nm (Figure 4B), and there was no observable difference in the fluorescence spectra of the native and 0.1−10 mM NAI-modified MSPs (data not shown), suggesting that the modification had very little effect on the structure and conformation of MSP. This was further confirmed by far-UV CD (Figure 5), which revealed that both native and 10 mM NAI-modified MSP displayed similar spectral features with a positive peak at 195 nm and a flattened negative peak at 206 nm, suggesting that MSP with ~5.2 Tyr residues modified by NAI was not altered in terms of its basic secondary structure.

A further increase in the NAI concentration (to 20 mM) led to acetylation of an additional 1.2 (i.e., 1−2) Tyr residues for a total of ~6.4 acetylated Tyr residues. Under normal conditions, these Tyr residues are likely to be superficially buried in MSP, since lower concentrations of NAI did not modify them. NAI concentrations ranging from 20 to 100 mM showed no further increases in the number of acetylated Tyr residues in MSP, implying that saturation had been reached and no further Tyr residues were accessible for acetylation. MSP with 6.4 modified Tyr residues lost its ability to rebind with urea/NaCl-washed PSII membranes

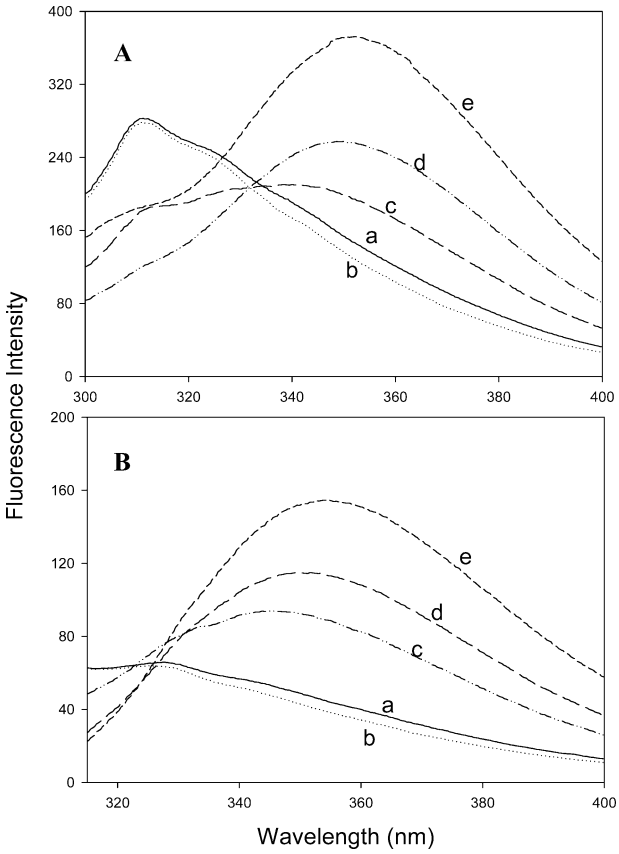



FIGURE 4: Fluorescence spectra of native MSP (a), renatured MSP after urea treatment (b), MSP with 6.4 tyrosines modified (c), MSP with 8 tyrosines modified (d), and MSP denatured by urea treatment (e) at 280 nm (A) and 295 nm (B). The spectra of a−d were measured in 10 mM phosphate buffer (pH 6.5) with a protein concentration of 20 $\mu$M, whereas the spectrum of e was measured under the same conditions with the addition of 8 M urea (all at 25 °C).

(Figure 2, lane 9) and also showed no reactivation of oxygen evolution (Table 1).

It is well-known that hydroxylamine deacetylates acetyltyrosine to regenerate their original functional groups at pH 7.5, but not acetylserine or acetylthreonine (*26*). Accordingly, hydroxylamine treatment was used to analyze whether NAI selectively modified the Tyr residues. The oxygen-evolving activity of NAI-modified proteins treated with 20 mM NAI was restored to near-control values after deacetylation with 1 M hydroxylamine (Figure 6, lane 3; Table 1), indicating that the acetylation/deacetylation of superficial Tyr residues accounts for the reversible abolition of reconstitution and oxygen evolution activity.

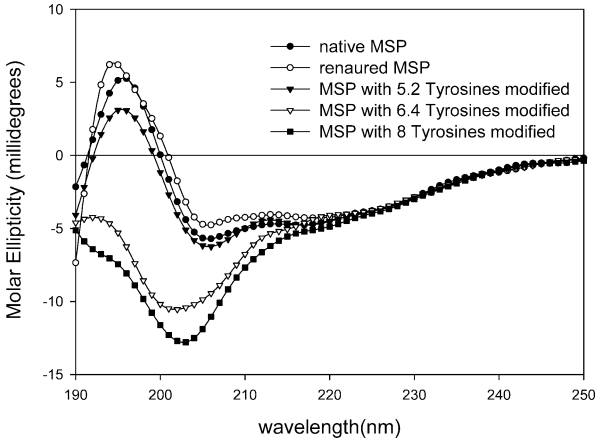



FIGURE 5: Far-UV circular dichroism spectra of native, NAI-modified, or renatured MSP after urea treatment. Protein concentration = 10 $\mu$M. $T$ = 25 °C. For details, see Materials and Methods.

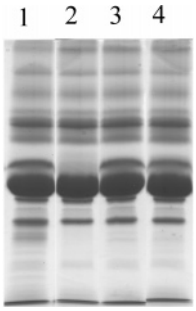



FIGURE 6: Reconstitution of various MSPs with 1 M urea/NaCl-washed PSII membranes. Lanes: 1, renatured MSP treated with 8 M urea; 2, MSP modified by 10 mM NAI in the presence of 8 M urea; 3, MSP with 6.4 modified tyrosine residues deacetylated with 1 M hydroxylamine; 4, MSP with 8 modified tyrosine residues deacetylated with 1 M hydroxylamine.

When the NAI concentrations were increased to 20 mM, the fluorescence emission spectra of MSP became broader and the peak wavelength showed a prominent red shift from 310 to 340 nm (Figure 4A). There was still evidence of a shoulder at about 310 nm. This was likely due to emission of tyrosines excited 280 nm, implying that some Tyr residues had not been modified by NAI. Figure 4B shows the changes of the fluorescence peak position for NAI-modified MSP when tryptophan was selectively excited at 295 nm. Following modification by 20 mM NAI, the 295 nm spectrum was significantly red shifted with a pronounced peak at about 345 nm, compared with the spectrum of the native protein. Since the single tryptophan residue, Trp241, is known to be buried in a hydrophobic region, this shift revealed that the hydrophobic region containing Trp241 had greatly changed, becoming hydrophilic.

Similarly, the CD spectra measured from 190 to 220 nm (Figure 5) changed significantly when the modification was carried out with 20 mM NAI. The ellipticity of MSP modified by 20 mM NAI was more negative than that of the native MSP or MSP modified by 10 mM NAI. Furthermore, the CD spectrum of MSP modified by 20 mM NAI contained a negative peak at 202 nm, which is often attributed to random coils. This significant change indicates that the percentage of secondary structure was dramatically changed when MSP was modified by 20 mM NAI, presumably due to modification of superficially buried Tyr residues. Together, these data suggest that the observed loss of rebinding and oxygen evolution activities in MSP with ~6.4 Tyr residues modified were primarily caused by a conformational change within MSP. Similarly, far-UV CD spectra have shown large changes in MSP secondary structure paralleled by a loss in reaction of oxygen evolution in experiments involving NBS modification of Trp241 (*19*) and reduction of the Cys28−Cys51disulfide bond (forming a disulfide bridge) (*9*, *31*). This implies that several key amino acid residues of MSP are important for determining the protein structure.

The fact that only about 6.4 Tyr residues in MSP had been modified by high doses of NAI led us to question whether some Tyr residues could be deeply buried in the interior of MSP. Accordingly, the NAI modification was carried out in the presence of 8 M urea, which could reversibly denature MSP for exposure of deeply buried Tyr residues. Under these conditions, 8.3 Tyr residues could be modified by NAI, which is very close to the theoretical value of 8.0. These results indicate that at least one Tyr residue in MSP was inaccessible to NAI until MSP was completely unfolded by 8 M urea. This residue forms the third group of tyrosine residues, the deeply buried residue(s). When MSP was modified by NAI in the presence of 8 M urea and then extensively dialyzed against 50 mM MES−NaOH (pH 6.5) for removal of the urea and other reagents, the MSP showed no rebinding ability or reactivation of oxygen evolution (Figure 6, lane 2; Table 1). However, deacetylation of NAI/urea-treated MSP with 1 M hydroxylamine restored the rebinding and oxygen evolving activities to near-control values (Figure 6, lane 3; Table 1).

In terms of fluorescence spectra, NAI/urea-treated MSP excited at 280 nm (Figure 4A) and 295 nm (Figure 4B) showed a further red shift, with emission maxima at about 350 nm, close to the peak spectrum of MSP unfolded by 8 M urea. Interestingly, the 310 nm shoulder observed at 280 nm in the spectra of MSP with 6.4 Tyr residues modified (treated with 20 mM NAI) was not visible in the spectra of MSP with 8 Tyr residues modified (treated with 10 mM NAI in the presence of 8 M urea). This finding is consistent with the conclusion that at least one Tyr residue was inaccessible to NAI until MSP was completely unfolded by 8 M urea.

In terms of CD spectra, when the modification was carried out with 10 mM NAI in the presence of 8 M urea, the ellipticity at 202 nm was lower than in samples modified with 20 mM NAI (Figure 5). This suggests that the NAI/urea modification induced significant conformational changes in the MSP, including an increase in the amount of random coils, possibly caused by modification of all 8 Tyr residues in MSP, indicating that the deeply buried tyrosine residue seems to participate in maintaining tertiary structure of the MSP. However, it is uncertain to know whether the deeply buried tyrosine residues play a decisive role in maintaining the secondary structure of MSP. In addition, reconstitution, oxygen evolution, fluorescence, and far-UV CD data showed that MSP treated with 8 M urea could be restored to the control values by extensive dialysis. Interestingly, the conformational changes shown by the CD spectra (Figure 5) of MSP modified by 10 mM NAI, 20 mM NAI, and 10 mM NAI/8 M urea were similar to those observed under conditions of varying medium pHs and environmental temperatures (*12*, *13*, *32*).

Among the eight Tyr residues in spinach MSP, seven Tyr residues are highly conserved in currently available amino

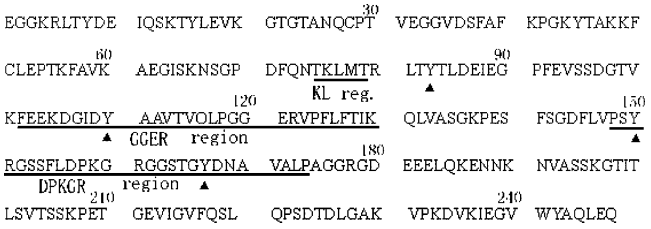
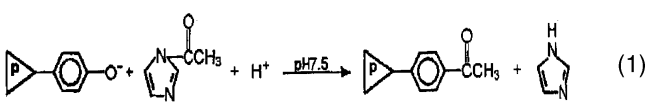



FIGURE 7: Summary of locations of KL, GGER, and DPKGR regions (underlined) as well as positions of Tyr83, Tyr110, Tyr150, and Tyr167 (with solid triangles beneath) in the spinach MSP. For details about the conserved regions within the multiple alignment, see De Las Rivas and Barber (*14*).

acid sequences from higher plants, except Tyr8, which is located in N-terminal region extending from the $\beta$-barrel domain of spinach MSP. Tyr83, Tyr110, Tyr150, and Tyr 167 happen to be close to or located in the KL, GGER, or DPKGR region (Figure 7), implicated to be exposed on the MSP surface for binding of MSP to PSII (*14*). Their topological sites are consistent with the fact that five tyrosine residues are easily modified by a low concentration of NAI. Different from these Tyr residues, however, Tyr16, Tyr45, and Tyr242 are within or close to the CC bridge or GEhhG region, which are considered for their importance in determining the structure of MSP (*14*). Tyr16, Tyr45, and Tyr242 might be probably those superficially or deeply buried Tyr residues in soluble MSP. Specifically, Tyr242 is next to the single tryptophan residue Trp241; as Trp241 and Tyr242 are buried in a hydrophobic $\beta$-sheet near the MSP C-terminus, which is critical for MSP rebinding and the recovery of oxygen evolution activity (*15*−*17*, *19*−*21*), it is reasonable to suppose that Tyr242 is very likely to be one of the superficially buried Tyr residues. However, further work will be needed to define the exact Tyr residues critical for MSP structure and function.

It is well-known that polar groups of ionizable amino acids mediate electrostatic interactions between various protein domains. NAI modification, which changes the polarity of the tyrosine side chain, can affect protein stability by changing various electrostatic interactions (see reaction 1). The phenolic OH group of tyrosine is expected to form two hydrogen bonds with neighboring residues (*33*). NAI modification replaces the phenolic OH group with an acetyl group, interrupting the ability of the Tyr residue to form hydrogen bonds. Among the changes in electrostatic interactions, hydrogen bond interruption may be the most important factor responsible for the conformational change in MSP induced by NAI modification. The ionization state of the Tyr residues in MSP is changed by NAI modification, but at neutral pH, the influence of ionization state change on MSP stability is very weak. When the initial ~5.2 Tyr residues were modified, the compact structure of MSP was not altered, suggesting that the electrostatic interactions of these exterior Tyr residues are not essential for maintaining the structure and function of MSP. In contrast to these exterior Tyr residues, superficially or deeply buried Tyr residues might contribute more hydrogen bonds that are actively participating in conformational foldings in MSP.




(1)

Ionizable residues such as tyrosine, lysine, and arginine play important roles in determining ligand binding, protein structure, and functional activity (*34*). With a p*K* value of 10.1 in solution, tyrosine is a protonizable amino acid residue in alkaline regions (*35*). When the medium pH was changed to 12, the structure of MSP was shown to become totally unfolded (*13*). A previous study suggested that in some cases the contribution of ionizable amino acids to protein stability arises from a small number of residues with anomalous p$K_a$s (*36*). The p$K_a$ value of MSP (determined from titration curves) is 11.7, markedly higher than that for L-tyrosine in solution (10.1), indicating that some or all of the tyrosine residues in MSP strongly interact with nearby residues (*20*, *36*). In this study, the fact that acetylation of 1−2 superficially buried Tyr residues induced a substantial conformational change in MSP implies that a few key tyrosine residues might be crucial for maintaining the secondary structure stability in MSP. These key tyrosine residues likely contribute to the pH dependence of MSP folding and unfolding.

Notably, our observation that a sudden prominent conformational change was observed only after the superficially buried tyrosine residues were acetylated suggests that the superficially buried tyrosine residues were exposed to the suspension medium only after the ~5.2 superficial Tyr residues were modified. It is very likely that the MSP conformational changes accumulated sequentially during acetylation, eventually leading to the larger structural change and increased accessibility of the superficially buried Tyr residues.

In summary, this study revealed that NAI acetylation could differentiate three groups of Tyr residues in soluble MSP. Different groups of tyrosine residues have different contributions to the binding and assembly of MSP into PSII membranes; 1−2 superficially buried Tyr residues play an important role in maintaining the functional conformation of MSP to rebind to PSII membranes.

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