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N-bromosuccinimide modification of tryptophan 241 at the C-terminus of the manganese stabilizing protein of plant photosystem II influences its structure and function

Yong Yu[a], Rong Li[a], Chunhe Xu[a,*], Kangcheng Ruan[b], Yunkang Shen[a] and Govindjee[c]

[a]*Shanghai Institute of Plant Physiology, Shanghai Institutes of Biological Sciences, The Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, P.R. China*
[b]*Shanghai Institute of Biochemistry, Shanghai Institutes of Biological Sciences, The Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200032, P.R. China*
[c]*Center of Biophysics & Computational Biology, University of Illinois, Urbana, IL 61801-3707, USA*
**Corresponding author, e-mail: xch@iris.sipp.ac.cn*

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Life on earth depends upon the ability of oxygenic photosynthesis to oxidize water to molecular oxygen. This process is catalyzed by water–plastoquinone oxido-reductase complex. In addition to the photosystem II (PSII) reaction core, it includes a manganese stabilizing protein (MSP) that plays an important regulatory role in the process in plants and algae. Tryptophan 241, located at the carboxyl-terminus of the MSP, is its sole tryptophan. Modification of MSP by N-bromosuccinimide (NBS) was carried out to explore the role of Trp241 in maintaining its structure and function. Data and arguments are presented to show that it is Trp241, not other tyrosines in MSP, that is involved in the modification and changes observed in this study. Further, the pH-dependence of the modification and the comparison of features of fluorescence spectra of MSP suggested that Trp241 is buried in the hydrophobic interior of the protein. Hydropathy analysis revealed that Trp241 is located in the middle of the hydrophobic region at the C-terminus of MSP. Circular dichroism spectroscopy showed that NBS modification of Trp241 dramatically modified the protein structure. The affinity of MSP to PSII decreased greatly after the modification of Trp241, and no oxygen-evolving activity was recovered after its reconstitution. This study provides a novel demonstration that Trp241 at the C-terminus hydrophobic region of the MSP is critical for maintaining appropriate structure and function of MSP.

Introduction

Photosystem II (PSII) of higher plants is a multi-subunit pigment-protein complex that catalyzes the light-driven reduction of plastoquinone, and the oxidation of water to molecular oxygen (Renger 1999). At least 6 intrinsic proteins are required for the oxygen evolution by PSII: CP47, CP43, the two reaction center subunits D1 and D2 proteins, and the two subunits of cytochrome b559 (Bricker and Frankel 1998, Ghanotakis et al. 1999). A 4-manganese atom cluster catalyzes the oxidation of water. Two other atoms, Ca^{2+} and Cl^{-}, are also required for the oxygen evolution. Three extrinsic proteins with apparent molecular masses of 33, 23 and 17 kDa, located at the lumenal surface of thylakoid membrane, are involved in the function of oxygen evolution in plants and algae. The 33-kDa protein is also called the manganese (Mn) stabilizing protein (MSP) as it functions to stabilize the Mn cluster at physiological conditions. Different treatments of PSII membranes lead to the release of the MSP from the membranes (Xu et al. 1995, Seidler 1996). This is accompanied by a simultaneous decrease of oxygen evolution, loss of two manganese atoms at low concentration of chloride anion. MSP has an ellipsoid shape in solution (Zubrzycki et al. 1998) and contains a low proportion of helix, and high proportion of β-sheet structure (Xu et al. 1994). Its molecular mass is 26.5 kDa (Zubrzycki et al. 1998), and its pI is 5.2 (Kuwabara and Murata 1979).

Abbreviations – CD, circular dichroism; Chl, chlorophyll; DMBQ, 2,6-dimethyl-*p*-benzoquinone; MES, 2-[N-morpholino]ethanesulfonic acid; MSP, manganese stabilizing protein; NBS, N-bromosuccinimide; PSII, photosystem II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Reconstitution of extrinsic MSP with the PSII membranes recovers, to a substantial extent, the activity of oxygen evolution (Ono and Inoue 1984). Evidence shows that conformation change may occur in the protein during reconstitution (Enami et al. 1998, Hutchison et al. 1998). Studies on the role of amino acid residues of MSP for the requirement of its binding to PSII are available (Seidler 1996, Bricker and Frankel 1998). After the first 16–18 amino acids at the N-terminal were removed by protease (Eaton-Rye and Murata 1989), the truncated MSP was unable to bind to the PSII complex. Two conserved cysteines (Cys28 and Cys51) in the MSP were suggested to form a disulfide bond providing correct structure for MSP to interact with PSII and restore the oxygen evolution activity (Tanaka and Wada 1988). Recently, roles of C-terminal amino acids of the MSP in the interaction with PSII have been studied. Introduction of 6 histidines at the C-terminus led to conformational changes in the MSP, and significant effects on the reconstitution process (Seidler 1994). Replacement of Val235 with Ala caused a cold-sensitive assembly of MSP with PSII (Betts et al. 1996). After the final 3–4 residues were truncated from the C-terminus of the MSP, or after Leu245 was substituted with Glutamate by mutation, its structure, rebinding capacity to PSII, and the recovery of oxygen evolution activity were dramatically decreased (Betts et al. 1998, Lydakis-Simantiris et al. 1999). We show the role of Trp241 in MSP with convincing evidence for influence on the structure and function of the MSP in the present work.

Covalent modifications of side chains of amino acids have been used to investigate the structure-function relationships of specific residues in MSP (Frankel and Bricker 1995, Miura et al. 1997). Among the 247 residues in the entire amino acid sequence of MSP (Oh-oka et al. 1986), only one tryptophan (Trp241) at the C-terminus is present. In this study, Trp241 of MSP was modified with N-bromosuccinimide (NBS), a frequently used chemical in the modification of tryptophan residues of proteins for its special reaction with tryptophan at acidic pH (Spande and Witkop 1967a, Lundblad 1995). In view of the possibility that tyrosines, present in MSP, may have been also modified, fluorescence spectra of MSP, and of a mixture of tyrosine and tryptophan with and without NBS, were measured. Data and arguments show that it was Trp241 that was modified and led to the effects discussed in this paper. Conformation change was discovered in MSP after NBS modification; meanwhile the rebinding of MSP to PSII and the recovery of oxygen evolution were obviously inhibited (for a short preliminary report, see Yu et al. 1999). Data indicate that Trp241 is critical for maintaining appropriate structure and function of MSP.

Materials and methods

Purification of extrinsic MSP

PSII membranes were isolated from market spinach leaves with the method of Berthold et al. (1981), with minor modifications (Xu et al. 1995). Freshly isolated PSII membranes were suspended in 0.4 *M* sucrose, 50 m*M* MES-NaOH, pH 6.2, 15 m*M* NaCl and 10 m*M* $MgCl_2$ (referred below as SMN medium). Chlorophyll (Chl) concentration was adjusted to 1.0 mg ml^{-1}. Membranes were treated with 1.5 *M* NaCl at 4°C for 1 h in room light. After centrifugation at 40000 *g* for 20 min, pellets were resuspended in the SMN medium and mixed with 2 *M* NaCl of the same volume. Then the suspension was immediately centrifuged at 40000 *g* for 20 min. Pellets were treated further by incubation in solution of 0.4 *M* sucrose, 50 m*M* MES-NaOH, pH 6.2, 15 m*M* NaCl, 10 m*M* $MgCl_2$ and 1 *M* $CaCl_2$ at 4°C for 30 min in the dark. After centrifugation at 40000 *g* for 20 min, the supernatant was saved and dialyzed overnight against 5 m*M* MES-NaOH, pH 6.2. Then, it was concentrated with an ultrafiltration device (Amicon 8050). The concentration of the MSP was calculated, as described by Eaton-Rye and Murata (1989), from the UV absorbance at 276 nm.

Chemical modification of MSP with N-bromosuccinimide

After each sequential addition of 1 µl 10 m*M* NBS (Fluka AG, recrystallized before use), the MSP suspension (12 µ*M*, 1 ml) was stirred for 2 min. Then, ultraviolet (UV) absorption spectra were obtained with a Shimadzu UV-3000 spectrophotometer. The decrease of the absorption at 280 nm was monitored until no further decrease took place. The number of the modified tryptophan in each protein was calculated by using the formula of Spande and Witkop (1967a). The pH of the MSP suspension was adjusted to pH 2.5 by the addition of 50% (v/v) acetic acid.

Measurement of the fluorescence spectra

Fluorescence emission spectra of the MSP were measured with a Hitachi F4010 fluorescence spectrophotometer. The concentration of MSP was 7.5 µ*M*. Excitation wavelength was set at 280 nm to excite both tryptophan and tyrosine, or at 295 nm to excite tryptophan. The NBS-modified MSP was dialyzed against 50 m*M* MES-NaOH, pH 6.2 for 3 h for the 'renaturation' of the protein before the measurement.

Hydropathy plot

A hydropathy plot was made to show the hydrophobic property of amino acids around Trp241, using the normalized consensus hydrophobicity values of amino acids given by Eisenberg et al. (1984). For the hydropathy calculations, a window size of one was used. The curve was smoothed by a moving average of three consecutive values.

Reconstitution of MSP with the PSII membranes

PSII membranes, frozen in liquid nitrogen, were thawed and diluted to 1.0 mg Chl ml^{-1} with SMN medium. Pellets were collected after a centrifugation at 40000 *g* for 20 min, and were resuspended with SCN_{low} solution (0.4 *M* sucrose, 10 m*M* $CaCl_2$, 10 m*M* NaCl and 50 m*M* MES-NaOH, pH 6.2)

to 1.0 mg Chl ml^{-1}. The suspension was treated with 2.6 *M* urea/0.2 *M* NaCl for 30 min at 4°C in the dark. Pellets were resuspended with the SCN_{high} solution (the same as SCN_{low} solution except that the concentration of NaCl was 180 m*M*), after a centrifugation at 40000 *g* for 20 min.

Before reconstitution, unmodified MSP (kept at pH 2.5, for the same time that was used for the NBS modification of MSP) and NBS-modified MSP were dialyzed separately against 50 m*M* MES-NaOH, pH 6.2 for 3 h. The precipitates, produced in the dialysis, were removed after centrifugation at 4000 *g* for 10 min. Then, the protein concentration was determined. For the NBS-modified protein, the absorption was corrected by taking into account the decrease caused by the modification. For reconstitution, the concentrations of urea/NaCl treated PSII membranes were adjusted to 0.1 mg Chl ml^{-1}. The MSP was added into the reaction medium to obtain a desired protein-to-PSII membranes ratio (details are provided in the Results section). PSII membranes and the MSP were incubated at 4°C for 30 min in the dark, and then centrifuged at 40000 *g* for 20 min. The pellets were washed twice with the SCN_{low} solution to remove the loosely bound MSP.

Measurement of the oxygen evolution activity

The oxygen evolution activity was measured with a Clark-type oxygen electrode in the SCN_{low} solution at 25°C. The chlorophyll concentration in the reaction medium was 10 μg ml^{-1}. 0.8 m*M* 2,6-dimethyl-*p*-benzoquinone (DMBQ) was used as the artificial electron acceptor.

SDS-PAGE analysis of protein content

Protein content was analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the system of Laemmli (1970) containing 6 *M* urea. A slab gel containing 5 (stacking gel) and 13.75% (resolving gel) acrylamide was used. The densitogram of the gel stained in Coomassie Brilliant blue R-250 was obtained with a Digital Imaging System (IS-1000), and the relative amounts of MSP were determined by integrating the peak areas.

Circular dichroism (CD) spectroscopy of MSP

The secondary structure of MSP was examined with far-UV CD spectroscopy. pH of both the unmodified and the NBS-modified MSP solutions were changed from 2.5 to 6.2 before measurement, through extensive dialysis in 10 m*M* KH_2PO_4-K_2HPO_4 buffer (pH 6.2). After the MSP solution was filtered through a polyethersulfone membrane (0.2 nm), the CD spectra was measured by a Jasco J-715 spectropolarimeter at 22°C. The concentration of MSP was adjusted to 10 μ*M* before each measurement. The cell length was 1 mm. Data were collected every 0.5 nm with a 1.5-nm bandwidth and 1-s time constant. Scan speed was 10 nm min^{-1}. Four scanning spectra were averaged and the data were linearly smoothed ± two points.

Results

Trp241 is specifically modified by NBS at low pH and is normally located in the hydrophobic interior of MSP

Reaction of NBS with tryptophan is a useful method for obtaining site-specific chemical modification in proteins (Spande and Witkop 1967a, Lundblad 1995). This method is based upon the fact that after the oxidation with NBS, indole chromophore of tryptophan, absorbing strongly at 280 nm, is converted to oxindole, which has a major absorbance at 250 nm. Since only one tryptophan is present in MSP (Oh-oka et al. 1986), NBS is expected to modify this sole tryptophan.

Low pH has been frequently used to facilitate the NBS modification of the tryptophan buried in the interior of the protein, and to reduce the possibility of reaction between NBS and other amino acids (Lundblad 1995). When the oxidation is conducted in acidic medium with carefully controlled amount of NBS, reaction is usually restricted to modification of tryptophan residues (Spande and Witkop, 1967a). At pH 2.5, an obvious decrease at 280 nm (A_{280}) and a correlated increase at 250 nm of UV absorption were observed after progressive additions of 10 m*M* NBS (1 μl each time) into the suspension (Fig. 1A), representing the transformation of indole in tryptophan to oxindole. Using the empirical formula of Spande and Witkop (1967a), the number of modified tryptophan in each protein, calculated from the decrease at 280 nm, was about 0.7 at this pH, close to the theoretical value of 1.0. This ratio remained unchanged even when more acid or 8 *M* urea was added (data not shown), indicating that the tryptophan in MSP was practically fully modified at pH 2.5.

Tyrosine was also suggested to react with NBS besides tryptophan (Spande and Witkop 1967a). To check if tyrosine was also involved in our experiment and to evaluate the specificity of the reaction between NBS and tryptophan, a simulated experiment was designed. A mixture of free tryptophan and tyrosine in a ratio of 1:8, consistent with the ratio in MSP, was prepared in 5 m*M* MES-NaOH (pH 6.2) buffer. pH was then adjusted to 2.5, giving the final condition used in the MSP modification experiment. From the fluorescence spectrum measured, two emission peaks can be seen at 305 and 355 nm, characteristic for free tyrosine and tryptophan respectively (Fig. 1B, curve 1). After sequential addition of 0.3 μl of 10 m*M* NBS, an obvious decrease can be seen at 355 nm, where the characteristic peak of free tryptophan is located (Fig. 1B, curve 2). It disappeared completely after 1.2 μl 10 m*M* NBS was added, while the 305 nm fluorescence peak, belonging to tyrosine, was not affected at all (Fig. 1B, curve 5), indicating that NBS under carefully controlled conditions reacted with tryptophan with high specificity in our experimental conditions.

Furthermore, the pH dependence of the NBS modification allows us to judge whether the modified tryptophan is exposed to the exterior or buried in the interior of protein (Spande and Witkop 1967b). In general, the majority of the tryptophan molecules, located in the interior of the protein, are oxidizable at acidic pH, but unavailable at neutral pH. Similarly, Trp241 of the MSP was 'reluctant' to be modified

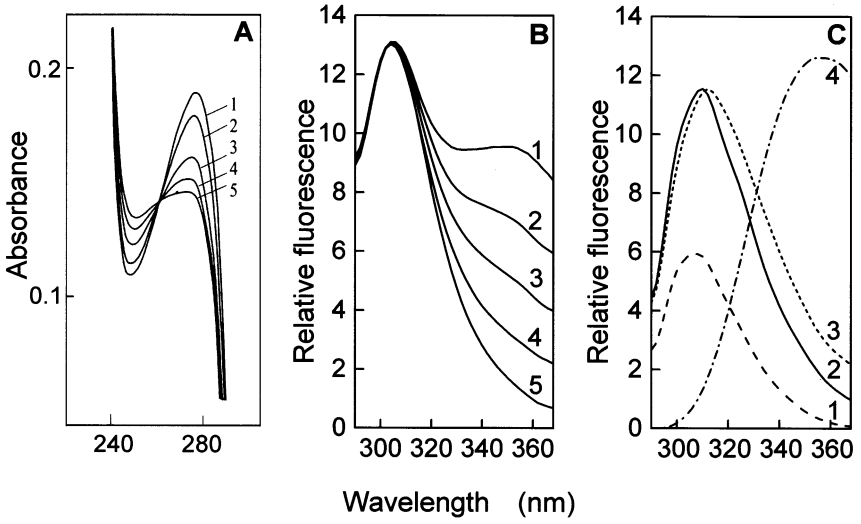

Fig. 1. UV absorption and fluorescence emission spectra of spinach MSP suspension. (A) UV (240–320 nm) absorption spectra, measured at pH 2.5. Curve 1: control; curves 2–5: after sequential additions of 1 μl 10 m*M* NBS. Protein concentration, 12 μ*M*. Measuring volume, 1 ml. (B) Fluorescence emission spectra of the tryptophan/tyrosine solution at pH 2.5. Excitation wavelength, 280 nm. Tryptophan concentration, 12 μ*M*. The molar ratio of tryptophan:tyrosine was adjusted to 1:8, as that in MSP. Curve 1: control; curves 2–5: sequential additions of 0.3 μl 10 m*M* NBS. (C) Fluorescence emission spectra of MSP. Excitation wavelength, 280 nm. Protein concentration, 7.5 μ*M*. Curve 1 (long dashes): measured with NBS-modified protein at pH 6.2; curves 2 (solid line) and 3 (short dashes): measured with unmodified (control) protein at pH 6.2 and 2.5, respectively. NBS modification was done at pH 2.5 and followed by a dialysis against 50 m*M* MES-NaOH, pH 6.2 for 3 h. The same procedures were used for the unmodified samples measured for curves 2 and 3, except that NBS was left out. Curve 4 (dashed-dot line), measured with free tryptophan in 50 m*M* MES-NaOH, pH 6.2. Free tryptophan concentration, 18 μ*M*.

at pH 6.2. Absorbance decrease at 280 nm was very small when MSP was incubated with NBS at pH 6.2 (data not shown). This indicates that the Trp241 is located in the intrinsic hydrophobic region of MSP. It will be shown later that the acidic pH used in this study did not damage the MSP.

To provide more evidence that Trp241 is specially modified by NBS and is buried in the hydrophobic interior of the MSP, fluorescence spectra of MSP were measured at pH 6.2 and 2.5. It is well known that in a protein, excitation energy is efficiently transferred from tyrosine to tryptophan, and normally fluorescence is emitted from tryptophan rather than tyrosine. The fluorescence emission peak of MSP (excitation wavelength, 280 nm) is at 310 nm at pH 6.2 (Fig. 1C, curve 2), consistent with earlier work (Tanaka et al. 1989, Shutova et al. 1997). This fluorescence should be emitted from Trp241. Free tryptophan in the polar solution fluoresces at about 355 nm, as shown by curve 4 in Fig. 1C. A shift of about 45 nm to the blue was clearly seen, when comparing the emission peak of the MSP (Fig. 1C, curve 2) with that of free tryptophan in polar solution (Fig. 1C, curve 4) at pH 6.2. It has been known that the emission peak of tryptophan blue-shifted when it was transferred into an apolar environment. The above fact also indicates that Trp241 is located in the hydrophobic interior of MSP. Change of the microenvironment of tryptophan from hydrophilic to hydrophobic caused the blue shift. Hydropathy plot (Fig. 2) showed clearly that Trp241 is located in a hydrophobic region, which happens to cover the last β-sheet, a previously predicted secondary structure unit existing in MSP (Xu et al. 1994).

Decreasing the medium pH to 2.5 caused not only a 5-nm shift of the emission peak to the longer wavelengths (compare curve 3 with curve 2 in Fig. 1C), but also a broadening of the spectrum on the long-wavelength side. This suggests that the low medium pH exposed some intrinsic residues including Trp241 to more hydrophilic region, enhancing reaction of Trp241 with NBS.

After reacting with NBS (adding 4 μl 10 m*M* NBS into 1 ml 7.5 μ*M* MSP solution at pH 2.5), tryptophan emitted no more fluorescence and the transfer of excitation energy between tyrosine and tryptophan was blocked. Therefore, fluorescence from tyrosine with a characteristic emission peak at 305 nm appeared. By comparing curve 1 with curve 2 in Fig. 1C, an apparent decrease of fluorescence of MSP, accompanied by an apparent shift of the emission peak from

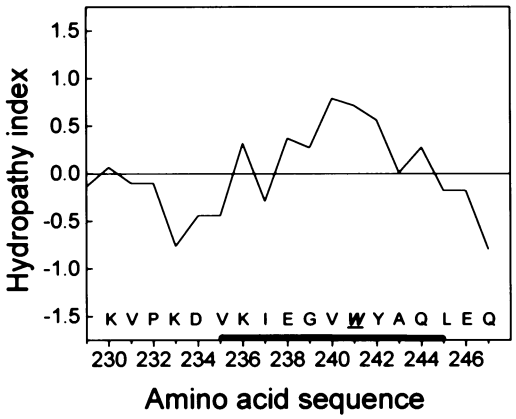

Fig. 2. Hydropathy plot for the amino acids that constitute the immediate surrounding of Trp241 in MSP. A thick bar on the X-axis shows where the last β-sheet of MSP was predicted to lie. Abbreviations of amino acids are printed above the X-axis, following the order of their sequence in MSP.

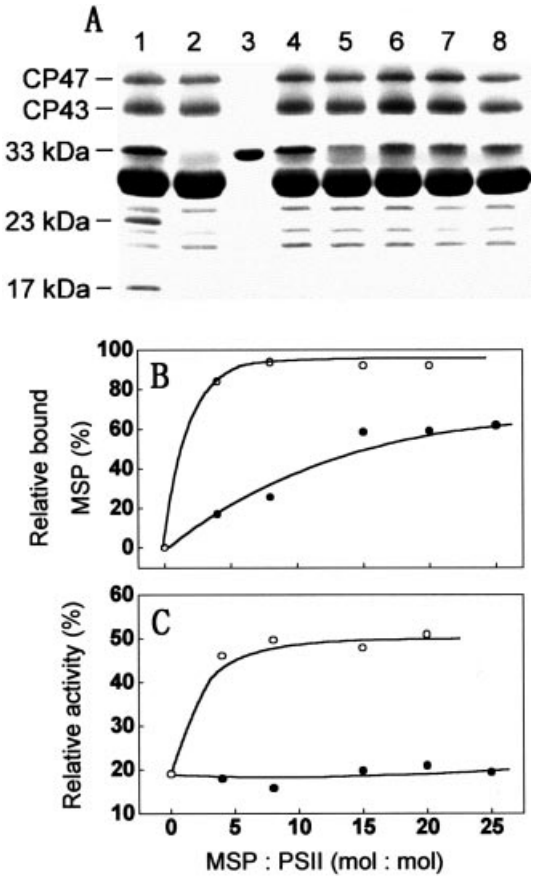

Fig. 3. Reconstitution of the NBS-modified MSP with PSII membrane. (A) SDS-PAGE analysis. Lane 1: control PSII membranes; lane 2: 2.6 *M* urea/0.2 *M* NaCl-washed PSII membranes, from which extrinsic proteins were depleted; lane 3: purified MSP; lane 4: reconstitution of unmodified MSP with the urea/NaCl-washed PSII membranes. The MSP:PSII ratio was kept as 8:1 mol. MSP/PSII were incubated at 4°C in the dark for 30 min, and were centrifuged afterwards and washed twice to remove loosely bound proteins; lanes 5, 6, 7 and 8: reconstitution of the NBS-modified MSP with urea/NaCl-washed PSII membranes, at ratios of 8, 15, 20 and 25:1 mol, respectively. (B) The amount of MSP retained with the PSII membranes after reconstitution. Solid circles, with NBS-modified MSP. Open circles, with unmodified MSP. (C) The relative oxygen-evolving activity of PSII complex after reconstitution. Solid circles, with NBS-modified MSP. Open circles, with unmodified MSP.

310 to 305 nm, was found. When the excitation wavelength was changed to 295 nm, a specific excitation wavelength for tryptophan, very little fluorescence emission was detected (data not shown). It is consistent with the previous conclusion that all tryptophan in the MSP were modified by NBS.

NBS modification of Trp241 affects the binding and function of MSP

NBS-modified MSP were dialyzed against 50 m*M* MES-NaOH, pH 6.2 to remove the remaining NBS. For the reconstitution experiment, MSP was incubated with the urea/NaCl treated PSII membranes at 4°C for 30 min in the dark. Loosely bound MSP was removed by washing twice with the SCN_{low} solution after the incubation. SDS-PAGE analysis was done after the reconstitution (Fig. 3A). Lanes 1, 2 and 3 are for protein content analysis of the control, Urea/NaCl-treated PSII membranes (lacking 33-, 23- and 17-kDa proteins) and purified MSP, respectively. Lane 4 shows the protein content of PSII membrane after reconstitution with unmodified MSP, when the MSP-to-PSII ratio was kept at 8:1 mol in the reconstitution system. Calculation of this ratio was done, based on the assumption that each PSII center has 260 chlorophyll molecules. The amount of the rebound unmodified MSP was judged to be 94% of that retained in the control (Fig. 3B). Fig. 3A also showed that the relative amount of MSP rebound to PSII decreased after the MSP was modified by NBS. When the ratio of protein-to-PSII was kept at 8:1 in the reconstitution system, the amount of NBS-modified MSP retained in the reconstituted PSII complex was $\sim$26% of that in the control (lane 5 in Fig. 3A, Fig. 3B). However, when the ratio of NBS-modified MSP to PSII was increased to 15:1, the amount of protein in the reconstituted PSII complex reached 60% of that in the control (lane 6 in Fig. 3A, Fig. 3B). The amount of NBS-modified MSP rebound saturated when this ratio reached 20:1 (lanes 7 and 8 in Fig. 3A, Fig. 3B). These results establish that the NBS-modified MSP binds with more difficulty to PSII.

Ono and Inoue (1984) showed that the oxygen evolution activity of PSII membranes, depleted of extrinsic proteins, can be restored to a large extent after reconstitution with MSP. In the present work, the oxygen evolution activity of PSII membranes depleted of all the three extrinsic proteins (33-, 23- and 17-kDa proteins) was 19% of that in the control, consistent with a previous work (Bricker 1992). The oxygen evolution activity was restored to 50% of that in the control, after the reconstitution of PSII membranes with the unmodified MSP (Fig. 3C and Table 1). Fig. 3 shows clearly that although the NBS-modified MSP do bind to PSII (Fig.

Table 1. Activity of oxygen evolution, measured after the reconstitution of the urea/NaCl-washed PSII membranes with the MSPs. Temperature, 25°C; chlorophyll concentration, 10 μg ml^{-1}; electron acceptor, 0.8 m*M* DMBQ. Data shown here are the average values of 3 experiments ± SE.

Samples	Retained 33-kDa protein (%)	Oxygen-evolving activity	
		(μmol O_2 [mg Chl]$^{-1}$ h^{-1})	(%)
Control PSII membranes	100	234 ± 9	100
Urea/NaCl-washed PSII membrane	~0	45 ± 4	19
Urea/NaCl-washed PSII + unmodified MSP (MSP: PSII = 8:1 mol)	94 ± 3	116 ± 5	50
Urea/NaCl-washed PSII + NBS-modified MSP (MSP:PSII = 20:1 mol)	59 ± 3	49 ± 5	21

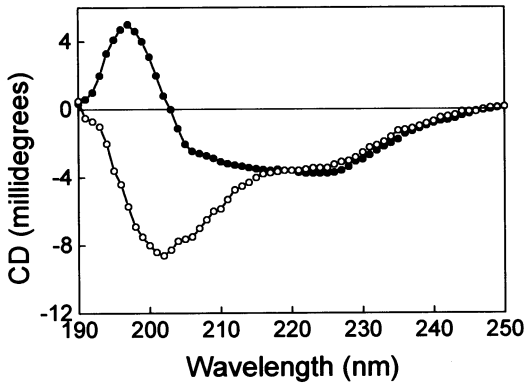

Fig. 4. Far-UV CD spectra of the NBS-modified and unmodified MSP. Data points were collected at protein concentration of 10 μ*M*, suspended in the 10 m*M* KH_2PO_4-K_2HPO_4 buffer, pH 6.2, 22°C. For experimental details, see Materials and methods. Solid circles, unmodified MSP. Open circles, NBS-modified MSP.

3B, solid circles), no recovery of oxygen evolution activity takes place (Fig. 3C, solid circles). Even with ~60% binding at a protein-to-PSII ratio of 20:1, oxygen evolution activity remains at the starting value of ~20% (Table 1 and Fig. 3C).

To exclude possible effects caused by pH change in the NBS modification procedure, reconstitution of PSII membranes with two kinds of control MSP was carried out. Two control protein samples were kept in the medium without NBS at pH 2.5 and 6.2 separately, for exactly the same time we had used for the NBS modification. Thereafter, both the samples were dialyzed against 50 m*M* MES-NaOH, pH 6.2, for 3 h, just as was done for the NBS-modification. Identical results were obtained for the reconstitution and for the oxygen-evolving restoration (data not shown). Thus, it is reasonable to conclude that no apparent effects were induced by the pH change in the NBS modification. We conclude that the major influence is on the modification of Trp241 of MSP with NBS.

NBS modification of Trp241 causes structural changes in MSP

Analysis of the solution structure of MSP after the NBS modification is indispensable for further correlating functional changes with structural transformation. Data from the far-UV CD spectroscopy was collected for this purpose. The CD spectra of control MSP (Fig. 4, solid circles), dialyzed from medium pH 2.5 back to 6.2, was similar to that of the native protein (data not shown; for examples, see Xu et al. 1994). This implies that no irreversible conformation changes were caused by the pH change in the process of NBS modification. After the NBS modification of Trp241, a very different CD spectra of MSP was observed (Fig. 4, open circles). When the wavelength was longer than 215 nm, there was no change in the CD spectra caused by the NBS modification. However, a significant change was observed below 215 nm. The ellipticity of the NBS-modified MSP was more negative than the control protein in this region. Negative, rather than positive, CD values in millidegree were obtained below 203 nm. Negative peak appears at 202 nm. These differences reflect an increased random coil in the protein's solution structure (Johnson 1990, Kelly and Price 1997), implying that the MSP structure became looser after the NBS modification. CD spectrum of NBS-modified MSP was similar to that measured with the Val235Ala mutation (Betts et al. 1996), Leu245Glu mutation, and the MSP with final 3–4 residues truncated at C-terminal (Lydakis-Simantiris et al. 1999). It indicates that some similarities may exist when modification is done near the C-terminus. Tentative correlation can be drawn between MSP secondary structure in solution and its capability to bind to PSII as well as the reactivation of the oxygen evolution.

Discussion

MSP, the manganese stabilizing protein, is responsible for the structural and functional integrity of the manganese cluster in oxygen evolution complex of PSII. Although it is an important subunit of the oxygen-evolving complex of plant and algal PSII, relatively little is known about its structural organization. Structural and functional data on the role of the N-terminus of MSP are available (Eaton-Rye and Murata 1989). The role of the C-terminus of MSP in binding and restoration of PSII activity has been also studied recently (Betts et al. 1998, Lydakis-Simantiris et al. 1999). In the present work, we studied a unique specific structural feature at the C-terminus of the protein. Trp241, the sole tryptophan within the entire protein sequence of MSP (Oh-oka et al. 1986), located in the middle of the last β-sheet near the C-terminus. It is conserved in all eukaryotes (Seidler 1996) and conservatively substituted by phenylalanine, a residue with a similar structure and character with tryptophan, in prokaryotes. Thus, single residue modification by NBS was easily and reliably carried out in this paper to explore the contribution of Trp241 to the structure and function of MSP.

Tryptophan, with the aromatic group, indole, is an amino acid that generally exists in the hydrophobic region of protein. The nitrogen atom on the indole group usually forms a hydrogen bond with other groups on the main or side chains. The modification of tryptophan might affect the nearby hydrogen bond and hydrophobic microenvironment. NBS modification of tryptophan oxidizes its indole group to oxindole (Spande and Witkop 1967a). In this study, the addition of NBS and other experimental conditions were well controlled to guarantee specific modification of tryptophan rather than tyrosine (Fig. 1B).

Trp241 was hardly modified by NBS at normal pH (6.2), but significantly at low pH (e.g. pH 2.5, see the absorption spectra of MSP, Fig. 1A). The pH-dependence of the NBS modification implies that (a) the modification of Trp241 depends upon partial unfolding of MSP, and (b) Trp241 is located in the hydrophobic interior of the MSP. Analysis of the fluorescence spectra of MSP and hydropathy plot provides additional evidence for this conclusion (Fig. 1C and Fig. 2).

Results also showed that NBS modification of Trp241 produced a series of interesting consequences for MSP. First, CD spectra showed that the secondary structure of

MSP in solution changed significantly after the NBS modification of Trp241 (Fig. 4). Further, the binding affinity of MSP to PSII and its ability to restore oxygen evolution were greatly inhibited, after the modification (Fig. 3). Trp241, located at the C-terminus, is critical for maintaining structure and function of MSP of PSII.

A hydrophobic core of MSP was first implied in the correlation of the structure to function in study of the pH-induced change of protein structure (Shutova et al. 1997). Several residues in the C-terminus of MSP were recently suggested to form a hydrophobic strand and be critical for the maintenance of the structure of MSP (Lydakis-Simantiris et al. 1999). Study of Trp241 modification provides a novel demonstration to support this tentative conclusion. Hydrophobic analysis in this study revealed that a hydrophobic region, including residues from 235 to 245, really exists at C-terminus of MSP (Fig. 2). It was also predicted to form the last β-sheet of the MSP (Xu et al. 1994). Trp241, with prominent hydrophobic index, lies right in the middle of this region. Correspondingly, the effect of NBS modification of Trp241 on the structure and function of MSP is larger than that of single amino acid mutation on Val235, or Leu245 (Betts et al. 1996, Lydakis-Simantiris et al. 1999).

In some cases, such as replacing Leu245 with Glu (Lydakis-Simantiris et al. 1999), truncating final 3–4 residues from C-terminus (Betts et al. 1998, Lydakis-Simantiris et al. 1999) and modifying Trp241 with NBS, less oxygen evolution activity was restored, although large amount of MSP rebound to the PSII. Both unspecific association and inactive rebound MSP might cause this inconsistency, but both would be intimately related to the appropriate conformation of protein. Evidence about conformational change, occurring during the binding of the MSP to PSII, and assuring the normal function of oxygen evolution, is known through FT-IR measurements (Hutchison et al. 1998) and intramolecular cross-linking studies (Enami et al. 1998). The intramolecular cross-linked MSP, with decreased flexibility, could rebind to PSII and stabilize the binding of manganese as effectively as the untreated protein but restored little oxygen evolution activity (Enami et al. 1998). Thus, one role of MSP in the oxygen evolution might be to provide an appropriate microenvironment for the proper functioning of oxygen evolution.

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