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Positive Charges on Lysine Residues of the Extrinsic 18 kDa Protein Are Important to Its Electrostatic Interaction with Spinach Photosystem II Membranes

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Abstract To determine the contribution of charged amino acids to binding with the photosystem II complex (PSII), the amino or carboxyl groups of the extrinsic 18 kDa protein were modified with N-succinimidyl propionate (NSP) or glycine methyl ester (GME) in the presence of a water-soluble carbodiimide, respectively. Based on isoelectric point shift, 4–10 and 10–14 amino groups were modified in the presence of 2 and 4 mM NSP, respectively. Similarly, 3–4 carboxyl groups were modified by reaction with 100 mM GME. Neutralization of negatively charged carboxyl groups with GME did not alter the binding activity of the extrinsic 18 kDa protein. However, the NSP-modified 18 kDa protein, in which the positively charged amino groups had been modified to uncharged methyl esters, failed to bind with the PSII membrane in the presence of the extrinsic 23 kDa protein. This defect can not be attributed to structural or conformational alterations imposed by chemical modification, as the fluorescence and circular dichroism spectra among native, GME- and NSP-modified extrinsic 18 kDa proteins were similar. Thus, we have concluded that the positive charges of lysyl residues in the extrinsic 18 kDa protein are important for its interaction with PSII membranes in the presence of the extrinsic 23 kDa protein. Furthermore, it was found that the negative charges of carboxyl groups of this protein did not participate in binding with the extrinsic 23 kDa protein associated with PSII membranes.

Key words extrinsic 18 kDa protein; circular dichroism; electrostatic interaction; chemical modification

In higher plants, the photosystem II complex (PSII) converts light energy to chemical energy, reduces plastoquinone and promotes photosynthetic oxygen evolution. Most components of this complex are intrinsic membrane proteins, and several extrinsic membrane proteins have also been shown to participate in PSII function. At the donor side of PSII, a manganese cluster, composed of four atoms, takes part in catalyzing water splitting and oxygen evolution. On the luminal side of PSII, three extrinsic proteins, with apparent molecular weights of 33, 23 and 18 kDa, play important roles in oxygen evolution *in vivo* and *in vitro*. Among these extrinsic proteins, the 33 kDa protein is particularly important for stabilizing the manganese cluster under physiological conditions [1,2]. The functions of the extrinsic 23 and 18 kDa proteins are intimately related to the unique requirement of Ca^{2+} and Cl^-, respectively, for oxygen evolution [3–5]. In cyanobacteria and red algae, these roles are instead performed by the 12 kDa extrinsic protein and cytochrome c550, rather than by the 23 and 18 kDa extrinsic proteins utilized in higher plants [6,7]. Circular dichroism (CD) and Fourier transform infrared spectroscopy studies revealed that the 18 kDa protein is composed primarily of α-helices,

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with little β-strand structure [8,9]. This was confirmed by the crystal structure demonstrating that this protein contains a four α-helix bundle [10].

Chemical modification is a useful method to investigate the contribution of positive or negative charges on proteins to electrostatic interactions with other proteins. Glycine methyl ester (GME), in the presence of the water-soluble 1-ethyl-3-3-dimethylamino propyl carbodiimide (EDC), selectively modifies the negatively charged carboxyl groups of aspartyl and glutamyl residues as well as free C-termini to uncharged methyl ester groups [11]. Conversely, N-succinimidyl propionate (NSP) selectively reacts with positively charged ε-amino groups of lysine residues and the α-amino groups of free N-termini, also resulting in uncharged methyl ester groups [12].

The binding domains of the 18 kDa protein to PSII as yet remain obscure, so the present study endeavored to examine this interaction through chemical modification of charged residues using NSP and GME. Our results demonstrate that lysyl residue ε-amino groups of the extrinsic 18 kDa protein are important for its interaction with PSII in the presence of the 23 kDa protein.

Materials and Methods

Purification of the 23 kDa and 18 kDa proteins

The 23 and 18 kDa proteins were prepared from spinach PSII complex, according to the method of Berthold *et al*. [13]. Samples were further diluted six-fold with 30 mM citric acid (pH 4.0), immediately loaded onto a Bio-Scale S column (Bio-Rad, Hercules, USA), then equilibrated with 30 mM citric acid (pH 4.0). The 23 kDa protein was eluted with a NaCl gradient of 0.35–0.7 M, and the 18 kDa protein was eluted with a NaCl gradient of 0.7–1.0 M. Purified proteins were dialyzed against 10 mM phosphate buffer (pH 6.5) before use. All procedures were carried out at 4 ºC. Protein concentration was calculated from the UV absorbance at 277 nm according to the method of Kuwabara and Suzuki [14].

Modification of the 18 kDa protein with GME

Chemical modification of the 18 kDa protein was carried out as follows: 37.75 mM of the 18 kDa protein was incubated with 100 mM GME (pH 6.2) containing 2 mM EDC at 25 ºC for 12 h in the dark [15,16]. The mixture was then concentrated and washed with 20 mM phosphate buffer (pH 6.5) containing 1 M NaCl by ultrafiltration to remove unreacted or electrostatically attached reagents. Finally, the sample was passed through a Sephadex G-25 column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM phosphate buffer (pH 6.5).

Modification of the 18 kDa protein with NSP

For modification of amino groups of the lysyl residues and free amino termini, 48 mM of the extrinsic 18 kDa protein was incubated with 2–8 mM NSP in 20 mM phosphate buffer (pH 6.5) at 25 ºC for 90 min in the dark. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 20 mM phosphate buffer (pH 6.5) to remove unreacted NSP.

Measurement of the fluorescence spectra

Tryptophan fluorescence emission spectra of native and modified 18 kDa proteins (20 μM) were measured with a 970CRT fluorescence spectrophotometer using an excitation wavelength of 295 nm. GME or NSP modified 18 kDa protein was dialyzed against 10 mM phosphate buffer (pH 6.5) for 8 h prior to analysis.

CD spectroscopy

Samples of the 18 kDa protein were extensively dialyzed against 10 mM phosphate buffer (pH 6.5), then filtered through a polyethersulfone membrane (0.2 μm) prior to analysis. The concentration of the extrinsic 18 kDa protein in the solution was adjusted to 10 M before measurement. CD spectra were taken using a Jasco J-715 spectropolarimeter (Jasco Corp., Tokyo, Japan) with a cell (1 mm in length) at 25 ºC. Data were collected every 0.1 nm (bandwidth, 1 nm; time constant, 1 s) at a scan speed of 10 nm/min. Four scanning spectra were averaged and data were linearly smoothed by the addition of four adjacent points.

Reconstitution of the modified 18 kDa protein with $PSII^-$ in the presence of the native extrinsic 23 kDa protein

PSII was treated with NaCl as previously described [17]. PSII was suspended in SM solution (0.3 M sucrose, 25 mM MES-NaOH, pH 6.5) to a chlorophyll (Chl) concentration of 1 mg Chl/ml. After centrifugation at 40,000 *g* for 20 min, pellets were resuspended in SM solution to reach 1 mg Chl/ml, then mixed with an equal volume of 2.4 M NaCl (final concentration 1.2 M) for 20 min at 4 ºC in the dark. The mixture was then immediately centrifuged at 40,000 *g* for 20 min. The resulting pellets were washed twice with SM solution, 10 mM NaCl and 50 μM EGTA, and suspended in the same buffer. The resulting prepara-

tion contained PSII without the native 23 kDa and 18 kDa proteins ($PSII^-$).

For the reconstitution experiment, $PSII^-$ was suspended in SM solution containing 10 mM NaCl and 50 μM EGTA before the Chl concentration was adjusted to 0.1 mg Chl/ml. The extrinsic 23 kDa protein and chemically-modified 18 kDa protein were added to the $PSII^-$ suspension in a molar ratio of 8:8:1 (23 kDa:18 kDa:$PSII^-$). The mixtures were incubated at 4 ºC for 30 min in the dark, then centrifuged at 40,000 *g* for 20 min. Pellets were washed twice with SM solution containing 10 mM NaCl and 50 μM EGTA to remove the loosely bound 23 kDa and 18 kDa proteins, then suspended in the same buffer.

Analysis of protein content

Protein content was analyzed by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18] containing 6 M urea. A slab gel containing 5% acrylamide stacking gel and an 11.75% acrylamide resolving gel were used. The gel was stained with Coomassie brilliant blue R-250 (CBB) and analyzed using a digital imaging system (IS-100, Ampha Innotech Corp., USA). The contents of the 18 kDa protein were determined by integrating the peak areas relative to the amount of native 18 kDa protein reconstituted with $PSII^-$ (defined as 100%).

Isoelectric focusing

Isoelectric focusing was performed on 5.5% polyacrylamide gel covering a pH range of 4.45–9.6 using 5% (*V*/*V*) ampholine (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were stained with 30% methanol containing 0.048% CBB and 10% acetic acid for visualization.

Results

Effects of GME or NSP modification on the isoelectric point of the extrinsic 18 kDa protein

NSP and GME selectively modify amino and carboxyl groups of the target protein, respectively, to uncharged methyl ester groups. As a result, the isoelectric point (pI) value of the protein will shift toward lower values upon reaction with increasing concentrations of NSP, and GME will raise the pI value. As anticipated, the pI of the extrinsic 18 kDa protein decreased from 9.0 to 6.0–4.8 or 4.5–4.8 when reacted with 2 or 4 mM NSP (**Fig. 1**, lanes 2–4). The pI increased from 9.0 to 9.4–9.6 when the protein was reacted with 100 mM GME (**Fig. 1**, lanes 2 and 5).

The extent of chemical modification was analyzed using a computer pI/M_r analysis tool [19]. The results were that the pI shift following reaction with 2 or 4 mM NSP resulted from the neutralization of 4–10 or 10–14 amino groups. The pI shift after reaction with 100 mM GME indicated that 3–4 carboxyl groups were modified. It is notable that the modified protein appeared as multiple bands or a much broader band, compared with the native protein, during isoelectric focusing (**Fig. 1**, lanes 3–5), implying that the modified protein species are heterogeneous with respect to the number of neutralized charged residues. This result was similar to those on the modification of the extrinsic 33 kDa or 23 kDa protein with NSP or GME [17,18].

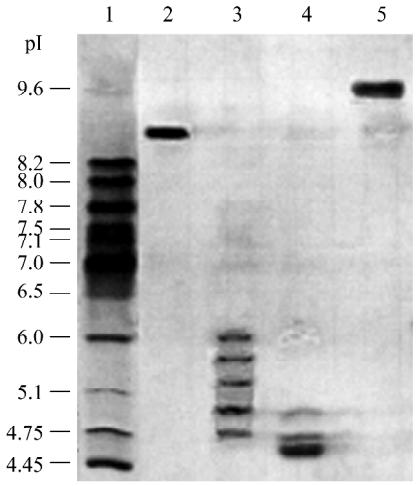

Fig. 1 Isoelectric focusing of the N-succinimidyl propionate (NSP)- or glycine methyl ester (GME)-modified 18 kDa protein
1, focusing marker; 2, the native 18 kDa protein; 3, the native 18 kDa protein modified by 2 mM NSP; 4, the native 18 kDa protein modified by 4 mM NSP; 5, the native 18 kDa protein modified by 100 mM GME in the presence of 2 mM EDC at 25 ºC for 12 h.

Effects of GME or NSP modification on fluorescence and CD spectra of the extrinsic 18 kDa protein

The intrinsic fluorescence of tryptophan residues in a protein is extremely sensitive to the local microenvironment and is widely used as a probe for changes in protein structure and conformation [20]. The single tryptophan amino acid (Trp71) present in spinach extrinsic 18 kDa protein is fully conserved throughout the PsbQ family [9]. This aromatic amino acid residue can be excited at a wavelength of 295 nm; and in the case of native extrinsic 18 kDa protein, an emission maximum is observed at 330 nm. The results in this study showed that the fluorescence spectrum of the native extrinsic 18 kDa protein shows a

maximum emission wavelength at 330 nm, while the 18 kDa protein modified by 4 mM NSP shows a maximum emission wavelength at 332 nm (**Fig. 2**), indicating that the surrounding microenvironment of Trp71 in the 18 kDa protein becomes more hydrophilic. However, this characteristic fluorescence emission spectrum is not obviously perturbed in either the GME- or NSP-modified extrinsic 18 kDa protein, suggesting that these modifications had little effect on overall structure and conformation.

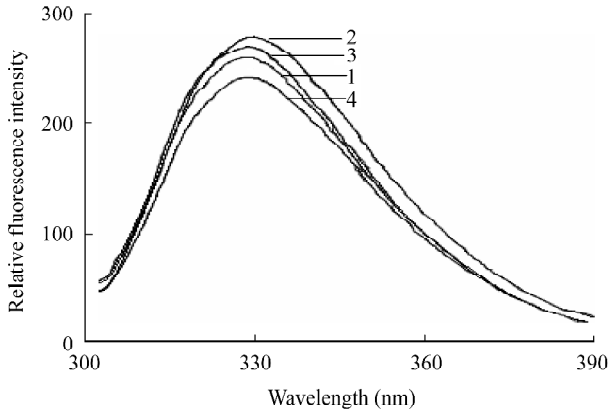

Fig. 2 Fluorescence emission spectra of the native, glycine methyl ester (GME)-modified or N-succinimidyl propionate (NSP)-modified 18 kDa protein

1, the native 18 kDa protein; 2, the native 18 kDa protein modified by 2 mM NSP; 3, the native 18 kDa protein modified by 4 mM NSP; 4, the native 18 kDa protein modified by 100 mM GME. Protein concentration is 20 μM. Excitation wavelength is 295 nm. For other details, see "Materials and Methods".

To further investigate the effects of chemical modification on the secondary structure of this protein, CD spectroscopy was also performed. Curve of the native protein (CK) was shown in **Fig. 3**, the CD spectrum of the native extrinsic 18 kDa protein is distinguished by a strong positive band near 193 nm and two strong negative bands near 209 and 219 nm, suggesting that α-helical structure is predominant, an observation consistent with previous studies [8–10]. CD spectra of modified extrinsic 18 kDa proteins displayed similar features, suggesting that the modification by GME or NSP did not markedly influence the basic secondary structure. Together, the tryptophan fluorescence and CD spectral analysis of native and modified 18 kDa proteins consistently support the conclusion that these modifications did not adversely affect overall protein structure.

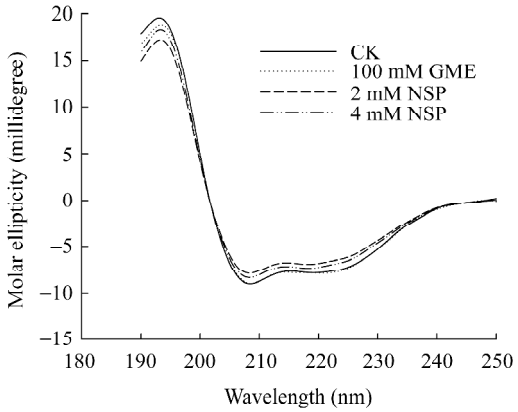

Fig. 3 Far-UV circular dichroism spectra of native (CK), glycine methyl ester (GME)-modified or N-succinimidyl propionate (NSP)-modified protein

Protein concentration is 10 μM, and the temperature is 25 ºC.

Effects of NSP or GME modification on the binding ability of the extrinsic 18 kDa protein to PSII⁻

In order to determine whether elimination of surface positive charges affected binding of the extrinsic 18 kDa protein to PSII⁻, the ability of the NSP-modified protein to bind PSII⁻ in the presence of the extrinsic 23 kDa protein was examined, and the reconstitution was analyzed by SDS-PAGE. Relative quantities of the extrinsic 18 kDa protein bound to PSII⁻ were determined by integrating the corresponding peak areas. The binding of native extrinsic 23 and 18 kDa proteins to PSII⁻ was complete (**Fig. 4**, lane 2; **Table 1**), whereas the binding of the NSP-modified extrinsic 18 kDa protein decreased with escalating NSP concentration (**Fig. 4**, lanes 4–6; **Table 1**) and was completely abolished by NSP treatment above 2 mM (**Fig. 4**, lane 5; **Table 1**). This suggests that the positive charges of lysine residues of the extrinsic 18 kDa protein are important for electrostatic interaction with PSII⁻. In the GME-modified extrinsic 18 kDa protein, although the negatively charged carboxyl groups were neutralized, the protein retained its ability to bind PSII⁻ with the presence of the extrinsic 23 kDa protein (**Fig. 4**, lane 3; **Table 1**). These results clearly indicate that positively charged residues are required, but negative charges on the surface of the extrinsic 18 kDa protein do not participate in binding with the extrinsic 23 kDa protein associated with PSII⁻.

Discussion

The present study clearly demonstrates that neutraliza-

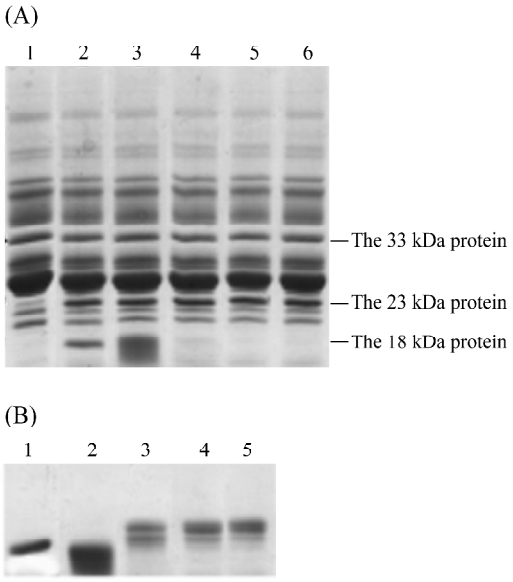

Fig. 4 Reconstitution of glycine methyl ester (GME)-modified or N-succinimidyl propionate (NSP)-modified 18 kDa protein with NaCl-washed protein-free photosystem II complex ($PSII^-$)

(A) 1, $PSII^-$; 2, $PSII^-$ reconstituted with the native 23 kDa and 18 kDa proteins; 3–6, $PSII^-$ reconstituted with the native 23 kDa protein and the 18 kDa protein modified by 100 mM GME (lane 3), 2 mM NSP (lane 4), 4 mM NSP (lane 5) or 8 mM NSP (lane 6). (B) 1, the native 18 kDa protein; 2–5, the native 18 kDa protein modified by 100 mM GME (lane 2), 2 mM NSP (lane 3), 4 mM NSP (lane 4) or 8 mM NSP (lane 5). $PSII^-$, 1.2 M NaCl-washed PSII without the native 23 kDa and various 18 kDa proteins.

Table 1 Rebinding ability of various 18 kDa proteins with the NaCl-washed photosystem II (PSII) membranes

Sample	Binding ability
$PSII^-$	0%
$PSII^-$ with the native 23 and 18 kDa protein	100%
$PSII^-$ with the native 23 and 18 kDa protein modified by 100 mM GME	100%
$PSII^-$ with the native 23 and 18 kDa protein modified by 2 mM NSP	0%
$PSII^-$ with the native 23 and 18 kDa protein modified by 4 mM NSP	0%

$PSII^-$, 1.2 M NaCl-washed PSII free of the 23 and 18 kDa protein. Binding ability was measured after reconstitution of $PSII^-$ with the native 23 kDa and various 18 kDa proteins. GME, glycine methyl ester; NSP, N-succinimidyl propionate.

tion of positively charged lysyl residues of the extrinsic 18 kDa protein with NSP [12] abrogates its binding to $PSII^-$ in the presence of the extrinsic 23 kDa protein, whereas, methyl-esterification of negatively charged carboxyl groups with GME [11] does not affect $PSII^-$-protein binding activity. We therefore conclude that positive charges on the surface of the extrinsic 18 kDa protein, but not negative charges, are important for its electrostatic interaction with $PSII^-$ in the presence of the extrinsic 23 kDa protein.

The extrinsic 18 kDa protein from *Spinacia oleracea* is composed of 159 amino acid residues; those germane to the present study include 9 aspartyl, 9 glutamyl, and 14 lysyl residues [21]. Based on observed pI changes, we estimate that 3–4 of the total 18 carboxyl groups in the extrinsic 18 kDa protein were modified by the conditions used in this study [100 mM GME (pH 6.2) and 2 mM EDC at 25 ºC for 12 h]. The pI shift was nearly saturated within 12 h even by modification in the presence of 4 mM and 8 mM EDC, implying that a number of the carboxyl groups on the extrinsic 18 kDa protein were not reactive with the chemical modification reagent. In spite of this extended reaction with GME, no significant effects were observed on the ability of the extrinsic 18 kDa protein to bind with $PSII^-$, supporting the conclusion that aspartyl and glutamyl residues of the extrinsic 18 kDa protein do not contribute to this interaction. In contrast, 4–10 amino groups of the 20 lysyl residues of the extrinsic 18 kDa protein were modified with NSP when the protein was treated with 2 mM NSP at 25 ºC for 90 min, indicating that these amino groups are more reactive than the aspartyl or glutamyl residues with respect to these chemical modification reagents.

The loss of $PSII^-$-binding activity of chemically-modified extrinsic 18 kDa protein could, in principle, be caused by either of two previously described mechanisms [15, 16]: (1) chemical modification may induce a conformational change of the protein, resulting in a protein structure that is no longer able to bind to $PSII^-$; or (2) the modified residues may participate directly in the electrostatic interaction of this protein to the $PSII^-$ in the presence of the extrinsic 23 kDa protein. We found no obvious structural or conformational differences between native and GME- or NSP-modified extrinsic 18 kDa protein by fluorescence and CD spectroscopy, therefore the former possibility appears unlikely. Thus the loss of binding activity of the NSP-modified extrinsic 18 kDa protein is most likely due to the neutralization of positively charged lysyl residues, not changes of protein structure. Consequently, we conclude that these positive charges are critical for binding to $PSII^-$ in the presence of extrinsic 23 kDa protein, whereas the negative charges of carboxyl groups do not participate in this activity.

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