Identification of Domains on the Extrinsic 33-kDa Protein Possibly Involved in Electrostatic Interaction with Photosystem II Complex by Means of Chemical Modification*

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The extrinsic 33-kDa protein of photosystem II (PSII) was modified with various reagents, and the resulting proteins were checked for the ability to rebind to PSII and to reactivate oxygen evolution. While modification of more than eight carboxyl groups of aspartyl and glutamyl residues with glycine methyl ester did not affect the rebinding and reactivating capabilities, modification of amino groups of lysyl residues with either N-succinimidyl propionate or 2,4,6-trinitrobenzene sulfonic acid resulted in a loss of re binding and reactivating capabilities of the 33-kDa protein. Moreover, the number of lysyl and arginyl residues susceptible to modification was significantly decreased when the protein was bound to PSII as compared with when it was free in solution, whereas the number of carboxyl groups modified was little affected. These results suggested that positive charges are important for the electrostatic interaction between the extrinsic 33-kDa protein and PSII intrinsic proteins, whereas negative charges on the protein do not contribute to such interaction. By a combination of protease digestion and mass spectroscopic analysis, the domains of lysyl residues accessible to N-succinimidyl propionate or 2,4,6-trinitrobenzene sulfonic acid modification only when the 33-kDa protein is free in solution were determined to be Lys4, Lys20, Lys66–Lys76, Lys101, Lys105, Lys186, Lys230–Lys236. These domains include those previously reported accessible to N-hydroxysuccinimidobiotin only in solution (Frankel and Bricker (1995) Biochemistry 34, 7492–7497), and may be important for the interaction of the 33-kDa protein with PSII intrinsic proteins.

The extrinsic 33-kDa protein of oxygen-evolving PSII complex is associated with the luminal surface of thylakoid membranes and plays an important role in maintaining functional binding of the manganese cluster that directly catalyzes the H2O-splitting reaction (for reviews, see Refs. 1–3). This protein can be removed from PSII membranes by washing with high concentrations of divalent cations (4) or urea plus NaCl (5), resulting in a substantial loss of the oxygen-evolving activity, which can be restored by rebinding of the protein (5–7). Removal of the protein also leads to a gradual liberation of two of the four manganese per PSII (8). Cross-linking of the protein with PSII intrinsic components by a water-soluble carbodiimide, which directly links amino and carboxyl groups in contact, prevents release of the protein upon CaCl2 or urea/NaCl wash or heat treatment, thereby stabilizing binding of the manganese cluster against these treatments (9–11). Based on these studies, the association of the 33-kDa protein with PSII has been proposed to involve electrostatic interactions between positive and negative charges on both the extrinsic protein and some PSII intrinsic subunits as well as hydrogen bonding (9, 12–14).

The 33-kDa protein from spinach is composed of 247 amino acid residues including 14 Asp, 21 Glu, 23 Lys, and 6 Arg (15), with an isoelectric point (pI) of 5.2 (16). Two Cys residues exist in the protein, which form a disulfide bond that has been shown to be important for maintaining the functional structure of the protein (17–19). Many attempts have been made to identify possible PSII intrinsic components with which the 33-kDa protein is associated. Trypsin digestion experiments have suggested that CP43, an intrinsic chlorophyll-binding 43-kDa protein, is shielded by the 33-kDa protein against proteolytic attack (20). A close association of the 33-kDa protein with CP47, another intrinsic chlorophyll-binding protein, has been demonstrated by cross-linking experiments with various bifunctional reagents (21–26). Photoaffinity cross-linking studies suggested that the protein is associated with D1 and D2, the...
two PSII reaction center proteins (27). The 33-kDa protein was also found to cross-link with the α subunit of cytochrome b$_{559}$ and the psb1 gene product (28). These results suggested that the extrinsic 33-kDa protein is associated with, or is in close proximity to, virtually all of the intrinsic PSI components.

There is, however, only very limited information concerning the residues on the 33-kDa protein responsible for its binding to PSI. Proteolytic cleavage of the first 16–18 N-terminal residues of the mature protein prevented its binding to PSI, suggesting the importance of the N-terminal region for binding (29). Site-directed mutagenesis of the protein in Synochocystis sp. PC 6803 showed that alteration of Asp$_{157}$ to Asn decreased the binding affinity of the protein to PSI (19), suggesting the possible involvement of this residue in either the interaction with PSI or in maintaining a functional structure of the protein required for binding. This is consistent with the results observed for the 33-kDa protein from spinach, where alteration of the corresponding Asp$_{157}$ reduced the oxygen-evolving activity (3, 30). In addition, in the spinach 33-kDa protein, alterations of Glu$_{104}$ and Asp$_{157}$ were also shown to slightly decrease the oxygen evolution, while changes of any one of the other conserved carboxylic residues did not affect the activity significantly (3, 30, 31).

Chemical modification of charged amino acid residues can easily alter surface charges on proteins and therefore is a useful technique to identify residues possibly involved in the electrostatic interaction between the 33-kDa protein and intrinsic PSI proteins. In an early study, Frankel and Bricker (32) modified the lysyl residues of the 33-kDa protein with NHS-biotin either in solution or on PSII membranes and showed that lysyl residues in domains Glu$_1$–Lys$_4$, Lys$_{20}$, Lys$_{101}$–Lys$_{105}$, Lys$_{159}$–Lys$_{186}$ were accessible to NHS-biotin only when the 33-kDa protein was released from the PSI membranes. They concluded, therefore, that these domains may be responsible for the interaction of the 33-kDa protein with PSI intrinsic proteins. In the present study, either negative charges of aspartyl and glutamyl residues (and free C terminus) or positive charges of lysyl (and free N terminus) and arginyl residues of the isolated extrinsic 33-kDa protein were modified using chemical modification techniques, and the abilities of the resultant modified protein to rebind to PSI and to reactivate oxygen evolution were examined. Data are presented showing that positive charges on the 33-kDa protein are essential for its electrostatic interaction with intrinsic PSI proteins, but elimination of surface negative charges on the protein had essentially no effect on its functional binding. Furthermore, the lysyl residues that are modified either when the 33-kDa protein is free in solution or when it is associated with PSI membranes, were determined by a combination of protease digestion and mass spectrometric analysis. From these results, the domains containing lysyl residues that are buried when the 33-kDa protein is associated with PSI but become exposed to bulk solution upon release of the protein from PSI membranes were determined. These domains may be located in regions that interact with PSI intrinsic components.

**MATERIALS AND METHODS**

**Preparation and Chemical Modifications—Oxygen-evolving PSI membranes (BBY PSI membranes)** were prepared from spinach chloroplasts with Triton X-100 as in Ref. 33, with slight modifications as described in Ref. 9. The isolated PSI membranes were suspended in medium A containing 40 mM Mes (pH 6.5), 0.4 mM sucrose, 10 mM NaCl, and 5 mM MgCl$_2$, and stored in liquid nitrogen until use. The extrinsic 33-kDa protein was extracted from the PSI membranes by 1 mM CaCl$_2$ treatment (4), incubated with 1 mM CaCl$_2$ for 3 h in the dark to suppress the activity of co-purified protease, dialyzed against 5 mM Mes-NeNaOH (pH 6.5), and then purified by column chromatography with a DEAE-Sepharose CL-6B column (Pharmacia Biotech Inc.) (34). The concentration of the 33-kDa protein was determined using an extinction coefficient of 16 mM$^{-1}$ cm$^{-1}$ at 276 nm (29).

Chemical modification of carbonyl groups on the purified 33-kDa protein was carried out in 100 mM GME (pH 6.2) containing 37.75 μM of the 33-kDa protein and 2 mM EDC at 25°C for 12 h in the dark. The reaction mixture was concentrated and washed with 1 mM NaCl and 20 mM Mes-NeNaOH buffer (pH 6.5) by ultrafiltration and electrostatically attached reagents and then passed through a Sephadex G-25 column equilibrated with 20 mM phosphate buffer (pH 6.5). For modification of the 33-kDa protein bound to PSI, the PSI membranes were washed with 1 mM NaCl to remove the 23- and 17-kDa proteins (35, 36) and then washed twice with and resuspended in 0.4 mM sucrose, 20 mM Mes-NeNaOH (pH 6.5) and then incubated with 1 mM CaCl$_2$, 25 mM Mes-NeNaOH (pH 6.5) to release the modified 33-kDa protein. The released 33-kDa protein was incubated with 1 mM CaCl$_2$, dialyzed, and then concentrated by ultrafiltration.

For modification of amino groups of lysyl residues and the free amino terminus of the 33-kDa protein, the purified protein (48 μM) was incubated in a reaction mixture containing 20 mM phosphate buffer (pH 6.5) and 0.5–8 mM N-succinimidyl propionate (NSP) at 25°C for 90 min in the dark. The reaction was stopped by passing the reaction mixture through a Sephadex G-25 column, dialyzed against 20 mM phosphate buffer (pH 6.5), which removed unreacted NSP from the reaction mixture. Site modification of the 33-kDa protein associated with PSI, the 23- and 17-kDa protein-depleted PSI membranes were incubated in medium A containing 8 mM NSP at 1 mg of Chl/ml for 15 h at 25°C. After modification, the PSI membranes were washed twice with medium A to remove the remaining NSP and treated with 1 mM CaCl$_2$ to release the modified 33-kDa protein. The released 33-kDa protein was concentrated as described above.

The lysyl residues and free N terminus of the 33-kDa protein were also modified with 3 mM or 20 mM TNBS in medium A for 15 h at 25°C at a protein concentration of 4.4 μM. After modification, the reaction mixture was passed through a Sephadex G-25 column, dialyzed against 20 mM phosphate buffer (pH 6.5), and then concentrated with the DEAE-Sepharose CL-6B column. For modification of the 33-kDa protein on PSI membranes, the 23- and 17-kDa protein-depleted PSI membranes were incubated with 4 mM TNBS at 1 mg of Chl/ml for 15 h at 25°C in medium A. The reaction was stopped by centrifugation, and the modified 33-kDa protein was released from the PSI membranes, dialyzed, and concentrated.

For modification of arginyl residues, the 33-kDa protein (33 μM) was incubated with 0.9–2.5 mM BD in 0.1 mM KCl, 0.1 mM H$_2$BO$_3$/NaOH (pH 8.4) for 70 min, and the reaction was stopped by passing the reaction mixture through the Sephadex G-25 column equilibrated in 20 mM phosphate (pH 6.5), 50 mM H$_2$BO$_3$. For modification of the 33-kDa protein bound to PSI, the 23- and 17-kDa protein-depleted PSI membranes were washed twice with and resuspended in 0.4 mM sucrose, 50 mM H$_2$BO$_3$, 20 mM NaCl and 50 mM Mes-NeNaOH (pH 7.5) and then incubated with 10 mM BD at 1 mg of Chl/ml for 2 h at 25°C (The pH of the reaction mixture was lowered to 7.5, as indicated at pH 8.4 led to a release of the 33-kDa protein from PSI). After incubation, the reaction mixture was washed twice with the same buffer containing boric acid and then treated with 1 mM CaCl$_2$, 50 mM H$_2$BO$_3$, 25 mM Mes-NeNaOH (pH 6.5) to release the 33-kDa protein. The released 33-kDa protein was incubated with 1 mM CaCl$_2$, dialyzed against 5 mM Mes-NeNaOH (pH 6.5), 50 mM H$_2$BO$_3$, and concentrated.

NSP was purchased from Wako Pure Chemicals (Tokyo, Japan); GME, EDC, and TNBS were purchased from Nacalai Tesque Chemicals (Tokyo, Japan).

**Reconstitution and Electrophoresis**—For reconstitution, native PSI membranes were washed with 2.6 mM urea, 0.2 mM NaCl to remove the three extrinsic proteins of 33, 23, and 17 kDa (5). The resultant PSI membranes were incubated with either the native or the modified 33-kDa proteins at a protein:Chl ratio of 0.6 (w/w), in medium A at 0°C for 30 min in the dark at a Chl concentration of 0.5 mg/ml. The reconstituted PSI membranes were collected by centrifugation at 35,000 × g for 10 min and then washed once with and resuspended in medium A. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (37) with a slab gel of 11.5% acrylamide containing 37.75 μM of the 33-kDa protein and 2 mM EDC at 25°C for 12 h in the dark. The reaction mixture was concentrated and washed with 1 mM NaCl and 20 mM phosphate buffer (pH 6.5). For modification of the 33-kDa protein bound to PSI, the PSI membranes were washed with 1 mM NaCl to remove the 23- and 17-kDa proteins (35, 36) and then washed twice with and resuspended in 0.4 mM sucrose, 20 mM Mes-NeNaOH (pH 6.5) and then incubated with 1 mM CaCl$_2$, 25 mM Mes-NeNaOH (pH 6.5) to release the modified 33-kDa protein. The released 33-kDa protein was incubated with 1 mM CaCl$_2$, dialyzed against 5 mM Mes-NeNaOH (pH 6.5), 50 mM H$_2$BO$_3$, and concentrated.
Interaction of the Extrinsic 33-kDa Protein with PSII

CBB in 30% methanol and 10% acetic acid. Oxygen evolution was measured at 25 °C with a Clark-type oxygen electrode in medium A, to which 5 mM CaCl₂ and 0.4 mM phenyl-p-benzoquinone were added. Chl concentration was determined by the method of Porra et al. (38).

**Protease Digestion**—The 33-kDa protein (20–30 nmol) modified with NSP either in solution or on PSII membranes was denatured in 1 M Tris-HCl (pH 8.4), 8 M urea, and 1 mM mercaptoethanol, precipitated, and then digested with the V8 protease. The digested protein mixture was monitored by a reversed phase HPLC every 8 h, and the digestion reaction was carried out for 2 days, after which the elution pattern was not further changed.

**HPLC Separation and Mass Spectroscopic Analysis**—The protease-digested protein mixture of NSP-modified 33-kDa protein was solubilized in water and directly applied to a MALDI-TOF mass spectrometer (Reflex; Bruker), with a matrix of either 2,5-dihydroxybenzoic acid or a-cyano-4-hydroxycinnamic acid. The mass of each measured peptide fragment was assigned to the known 33-kDa protein sequence.

The digestion mixture of TNBS-modified 33-kDa protein was separated with a reversed phase column (Bondasphere 5 μ C4300A, Waters Inc.) in an HPLC set-up (LC-9A, Shimadzu Inc., Japan). The column was eluted with a gradient of 0–75% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and the elution pattern was monitored at 210 nm for all peptides and 345 nm for peptides modified by TNBS. Each fraction was collected, dried, and resolubilized in 3 μl of 67% acetic acid, out of which 1 μl was mixed with the same volume of the matrix (a mixture of 1:1 (v/v) of glycerol and 3-nitrobenzyl alcohol) and analyzed with a fast atom bombardment mass spectrometer (JEOL JMS HX-110) at a voltage of 10 kV with xenon as the ionization gas. The resulting mass spectra were analyzed with a DA5000 data system and assigned to the known 33-kDa protein sequence.

The TNBS-modified peptides obtained from HPLC separation were also analyzed for their N-terminal sequence by Edman degradation of the peptides followed by sequence analysis with a protein sequencer (Applied Biosystems model 477A).

Amino acid composition of the native and BD-modified 33-kDa protein was analyzed by an HPLC set-up (LC-9A, Shimadzu Inc., Japan) following acidic hydrolysis of the proteins in the gaseous phase. The relative abundance of each amino acid in the 33-kDa protein was calculated with Leu as the standard.

**RESULTS**

**Modification of Negative Charges on the 33-kDa Protein**

The extrinsic 33-kDa protein has a pl of 5.2 (16) and is therefore negatively charged at neutral pH. Thus, we first attempted to modify the negatively charged carboxyl groups of aspartyl and glutamyl residues (and free C terminus) on the 33-kDa protein with 10% cold trichloroacetic acid and centrifuged, and the resulting precipitate was washed twice with acetone. The final precipitate was dried and then resolubilized in 0.1 ml of 0.1 M pyridine/acetic acid (pH 6.5). The denatured 33-kDa protein was digested first with 15 μg of Staphylococcus V8 protease at 37 °C for 4 h and then with another 15-μg protease at 37 °C overnight. The reaction was stopped by the addition of 10 μl of 100% acetic acid and dried.

The TNBS-modified 33-kDa protein was similarly denatured with 8 M urea, 1 M Tris-HCl (pH 8.4), 1 mM mercaptoethanol, precipitated, and then digested with the V8 protease. The digested protein mixture was monitored by a reversed phase HPLC every 8 h, and the digestion reaction was carried out for 2 days, after which the elution pattern was not further changed.

**Protein-CONHCH₂COOCH₃** + H₂O → **Protein-CONHCH₂COOCH₃** + **GME-modified protein**

**REACTION 1**

In this reaction, a negatively charged carboxyl group is replaced by an uncharged methyl ester group. As a result, the pl of the protein is anticipated to shift toward higher values. Fig. 1A shows that the modification indeed upshifted the pl value from 5.2 of unmodified protein (lane 1) to a value above 8.5 (lane 3). This change was estimated to result from modification of more than eight negatively charged carboxyl groups to uncharged groups, as calculated using a computer pl/M, tool (40).

**Fig. 1. GME-modified 33-kDa protein and its reconstitution with PSII.** A, isoelectric focusing of the native 33-kDa protein (lane 1), the 33-kDa protein modified by 100 mM GME on PSII membranes (lane 2), and the 33-kDa protein modified by 100 mM GME in solution (lane 3). The gel was stained with CBB. B, reconstitution of the native or GME-modified 33-kDa protein with urea/NaCl-washed PSII membranes. The native and reconstituted PSII membranes were analyzed by SDS-PAGE, and the 33-kDa protein was detected with an antibody raised against this protein from spinach. Lane 1, control BBY PSII membranes; lane U, urea/NaCl-washed PSII; lane 1, urea/NaCl-washed PSII reconstituted with the native 33-kDa protein; lane 2, urea/NaCl-washed PSII reconstituted with GME-modified 33-kDa protein. For other details, see “Materials and Methods.”

It should be noted here that the band of the modified protein appeared much broader than the native protein upon isoelectric focusing, implying that the resulting protein products may be composed of proteins with different numbers of carboxylic residues modified.

In order to determine whether elimination of surface negative charges affected binding of the 33-kDa protein to PSII, the ability of the GME-modified protein to rebind to urea/NaCl-washed PSII membranes was examined. Since the 33-kDa protein migrated much faster upon GME modification, resulting in a comigration of the modified protein with light-harvesting Chl apoproteins (data not shown), we used immunoblot analysis to detect the protein. As Fig. 1B shows, the GME-modified protein (lane 2) was able to rebind to the protein-depleted PSII membranes as effectively as the unmodified protein (lane 1), suggesting that elimination of the surface negative charges has no significant effect on binding of the protein to PSII. Table I shows that, upon rebinding of the GME-modified 33-kDa protein to urea/NaCl-treated PSII membranes, the oxygen evolution was restored to an extent comparable with that achieved by rebinding of the unmodified protein, indicating that the modified 33-kDa protein was fully functional upon rebinding.

These results clearly indicate that surface negative charges on the 33-kDa protein do not participate in its functional binding to intrinsic PSII proteins or are not important for maintaining the functional structure of the protein.

Fig. 1A also shows that GME modification of the 33-kDa protein associated with PSII membranes resulted in a similar shift in the pl value (lane 2) as seen upon modification of the protein free in solution (lane 3). This indicates that a similar number of the carboxyl groups are modified even when the 33-kDa protein is bound to PSII and suggests that the carboxyl groups susceptible to GME modification when the 33-kDa protein is free in solution are also accessible to GME when the protein is bound to PSII membranes. Thus, these carboxyl amino acid residues are not located in regions interacting with PSII intrinsic components. This agrees with the above finding that modification of these residues did not affect the binding and reactivation of the 33-kDa protein.
Modification of Positive Charges on the 33-kDa Protein: Lysyl Residues

Modification with NSP—The ε-amino groups of lysyl residues and α-amino group of free N terminus carry positive charges that can be selectively modified with NSP according to Reaction 2 (41). Upon this modification, positively charged amino groups of lysyl residues and the free N terminus are replaced by uncharged groups, which results in a downshift of the pl value of the protein. Fig. 2A shows that the pl of NSP-modified 33-kDa protein indeed shifted toward acidic pH with increasing NSP concentration, although here again the modified proteins showed broader bands due to mixing of proteins with slightly different pl values resulting from inhomogeneous modification. In Fig. 2B, we examined the rebinding capability of the NSP-modified proteins to urea/NaCl-washed PSII membranes. The protein was able to rebind to PSII after modification with NSP at a concentration of 0.5 mM, which shifted the pl from 5.2 to 4.9–5.1. Increasing the NSP concentration to above 2 mM, however, significantly decreased the rebinding capability of the modified protein. This decrease was paralleled by a decrease in the extent of restoration of oxygen evolution upon reconstitution of the modified protein. As Table I shows, a modified protein with pl of 4.4–4.7, which virtually cannot rebind to PSII, completely lost the reactivating capability. Since NSP-modified protein showed a weaker CBB-staining intensity and slower migration in the SDS-PAGE gel than the unmodified protein, we determined the amount of the modified proteins rebound by releasing them with urea/NaCl wash and comparing the staining intensities of the released proteins on the gels with the intensities of the known amounts of equally modified proteins. The amount of the rebound protein determined in this way showed a good correlation with oxygen evolution restored (Fig. 3), irrespective of the degree of the NSP modification. This indicates that loss of the reactivating capability of the NSP-modified proteins was caused directly by loss of their rebound, which in turn suggests that the modified proteins, when rebound, are fully functional and that there is apparently no nonspecific binding of the modified protein.

**TABLE I**

Reactivation of oxygen evolution by reconstitution of the 33-kDa protein modified with various reagents to urea/NaCl-washed PSII membranes

<table>
<thead>
<tr>
<th>Samples</th>
<th>pI</th>
<th>Oxygen evolution</th>
<th>Reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PSII membranes</td>
<td>5.2</td>
<td>565</td>
<td>100</td>
</tr>
<tr>
<td>Urea/NaCl-washed PSII</td>
<td>7.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSII reconstituted with</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified 33-kDa protein</td>
<td>5.2</td>
<td>371</td>
<td>100</td>
</tr>
<tr>
<td>GME-modified 33-kDa protein</td>
<td>&gt;5.5</td>
<td>373</td>
<td>101</td>
</tr>
<tr>
<td>0.5 mM NSP-modified 33-kDa protein</td>
<td>4.9–5.1</td>
<td>347</td>
<td>92</td>
</tr>
<tr>
<td>2.0 mM NSP-modified 33-kDa protein</td>
<td>4.5–4.9</td>
<td>167</td>
<td>32</td>
</tr>
<tr>
<td>4.0 mM NSP-modified 33-kDa protein</td>
<td>4.4–4.8</td>
<td>95</td>
<td>8</td>
</tr>
<tr>
<td>8.0 mM NSP-modified 33-kDa protein</td>
<td>4.4–4.7</td>
<td>72</td>
<td>0.2</td>
</tr>
<tr>
<td>3 mM TNBS-modified 33-kDa protein</td>
<td>4.5–4.8</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>20 mM TNBS-modified 33-kDa protein</td>
<td>4.3–4.7</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>0.9 mM BD-modified 33-kDa protein</td>
<td>4.9–5.1</td>
<td>185</td>
<td>38</td>
</tr>
<tr>
<td>1.5 mM BD-modified 33-kDa protein</td>
<td>4.4–4.7</td>
<td>82</td>
<td>4</td>
</tr>
<tr>
<td>2.5 mM BD-modified 33-kDa protein</td>
<td>4.4–4.7</td>
<td>61</td>
<td>0</td>
</tr>
</tbody>
</table>

In order to determine the exact pl value at which the protein loses its abilities to rebind to PSII and to reactivate oxygen evolution, the NSP-modified proteins with various pl values were separated by isoelectric focusing, and each band with different pl in the gel was cut out, extracted in 20 mM phos-
phosphate buffer (pH 6.5), and then reconstituted to urea/NaCl-washed PSII membranes. The results showed that the protein completely lost its rebinding and reactivating abilities at pH 4.7 (data not shown). A pI shift from 5.2 to 4.7 was calculated to correspond to elimination of about seven positive charges on the 33-kDa protein. As will be shown later, the number of domains of lysyl residues accessible to NSP modification in the 33-kDa protein bound to PSII is six less than in the protein free in solution. These results suggested that the lysyl residues modified by NSP when the 33-kDa protein is free in solution are largely located in regions interacting with PSII intrinsic components, and thus may be directly involved in binding to PSII.

Modification with TNBS—Since chemical modification of a specific residue depends not only on the position of that residue in the protein but also in some cases on the chemical reagents used, we used another reagent, TNBS, to modify the amino groups of the lysyl residues and free N terminus of the 33-kDa protein. TNBS modifies the amino groups of lysyl residues and free N terminus according to Reaction 3 (42). Like NSP, TNBS also removed positive charges of the amino groups and thus lowered the pI value of the 33-kDa protein (Table I). Fig. 4 shows reconstitution of the TNBS-modified 33-kDa protein with urea/NaCl-washed PSII membranes. Upon modification of the 33-kDa protein with 3 mM TNBS, the 33-kDa protein completely lost its ability to rebind to the protein-depleted PSII membrane. This leads to a complete loss of the reactivation of oxygen evolution by the modified protein, as shown in Table I. Modification of the 33-kDa protein with TNBS at 3 mM resulted in a decrease of pI from 5.2 to 4.5–4.8, which corresponds to elimination of seven or eight positive charges of the amino groups on average. This is in agreement with the results of NSP modification and also roughly agrees with the number of domains that become inaccessible to TNBS when the 33-kDa protein is bound to PSII (see below). These results strongly suggest that lysyl residues (and/or the N terminus) of the 33-kDa protein are important for the electrostatic interaction between the protein and PSII intrinsic components.

**Modification of Positive Charges on the 33-kDa Protein:**

Arginyl Residues

In addition to the amino group of lysyl residues, the guanidino group of arginyl residues also carries a positive charge. The positive charges of guanidino groups of the arginyl residues on the 33-kDa protein were converted to negative charges by modification of the 33-kDa protein with BD in the presence of boric acid, according to Reaction 4 (43). Fig. 5 shows rebinding of the BD-modified 33-kDa protein to urea/NaCl-washed PSII. While the 33-kDa protein was still able to rebind to PSII upon modification with BD at lower concentrations, its re-binding capability decreased upon modification with increasing BD concentration. When the 33-kDa protein was modified at BD concentrations higher than 1.5 mM, it was no longer able to rebind to PSII. Table I shows that the reactivation of oxygen evolution by the modified 33-kDa protein parallels its re-binding capability, implying that modification of positive charges of arginyl residues primarily inhibited the binding of the 33-kDa protein, which then resulted in the loss of reactivation of oxygen evolution.

Because the BD-modified arginyl residues are unstable in the absence of boric acid (43), it was impossible to estimate the number of modified arginyl residues by isoelectrophoretic analysis. Therefore, we determined the number of modified arginyl residues by analyzing the amino acid composition of the BD-modified protein. In the native 33-kDa protein, there are six arginyl residues, of which three were modified at 0.9 mM BD, whereas four were modified at 1.5 mM BD (Table II). In addition, we modified the 33-kDa protein bound to PSII membranes with BD and determined the arginyl residues that were modified. Table II shows that no arginyl residues were modified by BD when the 33-kDa protein was bound to PSII. These results suggest that the arginyl residues modified by BD in solution become shielded from contact with bulk solution upon binding of the protein to PSII, which in turn implies that these residues are located in regions of the protein in contact with PSII intrinsic proteins.

**Fig. 4.** Reconstitution of the native or TNBS-modified 33-kDa protein with urea/NaCl-washed PSII membranes. The PSII membranes were analyzed by SDS-PAGE, and the gel was stained with CBB. Lane B, control BBY PSII membranes; lane U, urea/NaCl-washed PSII; lane 1, urea/NaCl-washed PSII reconstituted with the native 33-kDa protein; lanes 2 and 3, urea/NaCl-washed PSII reconstituted with the 33-kDa protein modified by TNBS at 3 mM (lane 2) and 20 mM (lane 3). Lanes 1s–3s are the supernatants obtained after reconstitution as in lanes 1–3.
Identification of Lysyl Residues Possibly Involved in Binding of the 33-kDa Protein to PSII

The above results have shown that, while loss of negative charges did not affect binding and reactivating capabilities of the 33-kDa protein, loss of positive charges of either lysyl or arginyl residues led to a significant decrease in the re-binding capability and thereby reduced the reactivating capability of the 33-kDa protein. In order to identify the lysyl and arginyl residues possibly responsible for loss of the binding to PSII membranes of the 33-kDa protein, we modified the protein either in its free form (in solution) or in its bound form (on PSII membranes) and attempted to determine the location of lysyl and arginyl residues that are modified, by digesting the modified protein with *Staphylococcus* V8 protease followed by determination of the mass of the resultant peptide fragments with mass spectrometer. From the results obtained, the domains on the 33-kDa protein that contain lysyl residues modified only in solution but not on PSII membranes were determined; these domains may be located in regions interacting with PSII intrinsic components. Since the BD molecule is easily dissociated from arginyl residues due to the instability of the BD-modified arginyl residues in the absence of boric acid, we were unable to detect arginyl residues on the 33-kDa protein that were modified by BD in solution. (Note that there were no arginyl residues susceptible to BD modification when the 33-kDa protein was bound to PSII.) In the following experiments, only results for modification of amino groups of lysyl residues and free N terminus were presented.

Modification with NSP—The 33-kDa protein modified with 8 mM NSP either in its free form (in solution) or when it was bound to NaCl-washed PSII membranes, was digested with *Staphylococcus* V8 protease, and the resulting peptide mixture was analyzed directly by a MALDI-TOF mass spectrometer. Since whether a peptide fragment can be detected by the MALDI-TOF mass spectrometer depends in some cases on the matrix employed, two different matrices were used; one was 2,5-dihydroxybenzoic acid, and the other was α-cyano-4-hydroxycinnamic acid. This led to a more complete identification of the peptide fragments resulting from the V8 protease digestion of the 33-kDa protein. Peptide fragments yielded were assigned to the known amino acid sequences within a 0.2% mass error. Modification of the amino group with each NSP molecule results in an addition of an N-propionyl group, which corresponds to an increase of 56.0 Da in the molecular weight. Table III shows the results of peptide assignment obtained from V8 protease digestion of the 33-kDa protein that was modified with 8 mM NSP in solution. In total, there were 41 peptides identified ranging in mass from 517.9 to 4581.4 Da. Of these peptides, eight were modified with one molecule of NSP (Glu1–Asp9, Pro54–Glu62, Ser140–Asp168, Ser140–Glu181, Ser140–Glu182, Ser140–Glu183, Leu184–Glu187, and Asp188, Glu209), whereas five peptides were modified either with one or two molecules of NSP (Gly33–Glu62, Gly63–Glu87, Lys105–Glu139, Arg122–Glu139, and Leu227–Glu238), whereas five peptides were modified either with one or two molecules of NSP (Gly33–Glu62, Gly63–Glu87, Lys105–Glu139, Arg122–Glu139, and Leu227–Glu238). These results indicate that residues Lys66, Lys76, Lys130, Lys137, Lys159, and Lys186 are accessible to NSP when the 33-kDa protein is modified in solution. Additionally, one amino group of the lysyl residues (or N terminus) in domains Glu1–Lys4, Lys56–Lys60, and Lys190–Lys207 and two amino groups of the lysyl residues in domains Lys41–Lys60, Lys105–Lys137, and Lys230–Lys236 are accessible to NSP modification when the 33-kDa protein is free in solution.

Table IV presents results obtained for digestion of the 33-kDa protein that was modified with 8 mM NSP on the 23- and 17-kDa protein-depleted PSII membranes. In total, 32 peptides were identified, which ranged in mass from 518.1 to 4524.8 Da. Of these peptides, six were modified with one molecule of NSP (Glu33–Glu53, Pro54–Glu62, Gly63–Glu87, Arg122–Glu139, Asp188–Glu209, and Leu227–Glu238), whereas five peptides were modified either with one or two molecules of NSP (Gly33–Glu62, and one peptide was modified with two molecules of NSP (Ser37–Glu62). These results indicate that one amino group of the lysyl residues in domains Lys41–Lys49, Lys56–Lys60, Lys66–Lys76, Lys130–Lys137, Lys190–Lys207, Lys230–Lys236, and two amino groups of the lysyl residues in domain Lys41–Lys60, were accessible to NSP when the 33-kDa protein was modified on PSII membranes. Among these domains, Lys137 was determined to be modified by NSP in domain Lys130–Lys137 by a postsource decay method of the MALDI-TOF mass analysis. Apparently, the numbers of domains modified when the 33-kDa protein is bound to PSII became much less in comparison with those when the protein is modified in solution; residues Lys130, Lys159, and Lys186 and one of the lysyl residues (and/or N terminus) in domains Glu1–Lys4, Lys56–Lys60, and Lys230–Lys236 were modified by NSP only when the 33-kDa protein was free in solution but not on PSII membranes. These results indicate that these domains become exposed to bulk solution when the 33-kDa protein is released from PSII membranes and therefore suggest that these domains are in regions interacting with PSII intrinsic proteins.

Modification with TNBS—The lysyl residues (and free N terminus) of the 33-kDa protein that are modified by TNBS in
corded at 345 nm, only peptide fractions carrying TNP were
PSII membranes. Since the elution pattern in Fig. 6 was re-
33-kDa protein modified with TNBS either in solution or
the separation pattern by HPLC of the V8 protease-digested
nal sequence of the resulting peptide fragments. Fig. 6 shows
phase HPLC, and determined either the mass or the N-termi-
V8 protease, separated the peptide fragments with a reversed
either in solution or on PSII membranes with
digested the 33-kDa protein that had been modified with TNBS
following protease digestion and separation by HPLC. Thus, we
detected. Nineteen such fractions were obtained from the di-
gested mixture of the 33-kDa protein modified with TNBS in
solution, whereas only one fraction was obtained from the
digested mixture of the 33-kDa protein modified with TNBS on
PSII membranes, implying that most of the lysyl residues
became buried and not accessible to TNBS upon binding of the
33-kDa protein to PSII membranes. The 19 fractions obtained,
however, do not necessary represent 19 peptides carrying dif-
ferent TNBS-modified lysyl residues (see below).

Table V shows the results of mass spectroscopy combined
with N-terminal sequence analysis of the peptide fractions
separated by HPLC. The peptide in the only fraction obtained
from V8 protease digestion of the 33-kDa protein modified with
TNBS on PSII membranes had a measured mass of 1379.4 Da, which is consistent with the predicted mass of Glu\(^1\)–Glu\(^{10}\) plus one molecule of TNBS (with an add-on mass of 211.0 Da). In the peptide Glu\(^1\)–Glu\(^{10}\), there are two amino groups, one is in the N terminus and the other is in the Lys4. However, N-terminal sequence analysis of fraction 1 revealed that the N terminus was blocked. Thus, the N-terminal a-amino group was concluded to be modified by TNBS when the 33-kDa protein is bound to PSII membranes as well as in solution.

Within the other 18 fractions obtained from V8 protease digestion of the 33-kDa protein modified with TNBS in solution, the mass of the peptides in fractions 9, 13, 17, 18, and 19 could not be determined by the fast atom bombardment mass spectrometer, presumably because these peptides had large masses and thus escaped from the detection of the mass spectrometer. We also tried to sequence the N termini of these peptides; only the N terminus of the peptide in fraction 13 could be sequenced, but the other fractions did not yield any significant phenylthiohydantoin signals. The sequence obtained from fraction 13 is VIGVFQSLQPSDTDLGAKVPXDV. This sequence corresponds to the sequence starting at Val\(^{213}\) in the 33-kDa protein. There are three lysyl residues in the peptide fragment from Val\(^{213}\) to the C terminus of the 33-kDa protein. There are three lysyl residues in the 33-kDa protein modified with NSP on PSII membranes depleted of the 23- and 17-kDa proteins, among which Lys\(^{230}\) was detected by the N-terminal sequencing. The mass of the peptides in fractions 9, 13, 17, 18, and 19 could not be determined by the fast atom bombardment mass spectrometer, presumably because these peptides had large masses and thus escaped from the detection of the mass spectrometer. We also tried to sequence the N termini of these peptides; only the N terminus of the peptide in fraction 13 could be sequenced, but the other fractions did not yield any significant phenylthiohydantoin signals. The sequence obtained from fraction 13 is VIGVFQSLQPSDTDLGAKVPXDV. This sequence corresponds to the sequence starting at Val\(^{213}\) in the 33-kDa protein. There are three lysyl residues in the peptide fragment from Val\(^{213}\) to the C terminus of the 33-kDa protein (Lys\(^{230}\), Lys\(^{231}\), and Lys\(^{236}\), among which Lys\(^{230}\) was detected by the N-terminal sequencing. Thus, the lysyl residues that were modified by TNBS in fraction 13 are most probably Lys\(^{230}\) and/or Lys\(^{236}\), although the possibility cannot be excluded that Lys\(^{230}\) was very weakly modified so that it could still be detected by the N-terminal sequencing. The mass of the peptides in the remaining 15 fractions were determined, among which fractions 3, 4, and 15 had masses that could not be assigned to a known sequence, probably due to nonspecific digestions of the V8 protease, which is often observed in such a prolonged digestion reaction as was conducted in the present study. From the measured masses of the remaining fractions, the residues or domains containing lysyl residues that were determined to be modified by TNBS in solution were Glu\(^1\), Lys\(^4\), Lys\(^{14}\), Lys\(^{20}\), Lys\(^{66}\)–Lys\(^{76}\), Lys\(^{101}\), Lys\(^{105}\), Lys\(^{159}\), and Lys\(^{230}\)–
Table V
Assignments for peptides separated by a reversed phase HPLC from a Staphylococcus V8 protease digest of the 33-kDa protein that was modified by 3 mM TNBS in solution

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Observed mass</th>
<th>Predicted mass</th>
<th>Change in mass</th>
<th>Peptide assignment</th>
<th>TNBS-modified domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1379.4</td>
<td>1379.3</td>
<td>0.01</td>
<td>Glu₁⁻Glu₁⁰ + TNP</td>
<td>Glu₁⁰⁻Lys⁴</td>
</tr>
<tr>
<td>2</td>
<td>1422.3</td>
<td>1423.2</td>
<td>-0.06</td>
<td>Glu₁⁻Glu₁⁰ + TNP + 2Na</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1418.9</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1913.9</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2516.5</td>
<td>2514.4</td>
<td>0.08</td>
<td>Pro¹⁵⁸⁻Glu¹⁶¹ + TNP</td>
<td>Lys¹⁵⁹</td>
</tr>
<tr>
<td>6</td>
<td>1237.7</td>
<td>1237.1</td>
<td>0.05</td>
<td>Ile¹¹⁻Glu¹⁸ + TNP + 2Na</td>
<td>Lys¹⁴</td>
</tr>
<tr>
<td>7</td>
<td>1590.3</td>
<td>1590.4</td>
<td>-0.01</td>
<td>Glu₁⁻Glu₁⁰ + 2TNP</td>
<td>Lys¹⁴⁻Lys⁴</td>
</tr>
<tr>
<td>8</td>
<td>2914.1</td>
<td>2913.1</td>
<td>0.03</td>
<td>Gly⁴⁰⁻Asp⁶⁶ + TNP</td>
<td>Lys⁹⁶⁻Lys⁹⁷</td>
</tr>
<tr>
<td>9</td>
<td>2536.7</td>
<td>2536.4</td>
<td>0.01</td>
<td>Ile⁹⁸⁻Asp¹⁰⁶ + 2TNP</td>
<td>Lys¹⁰¹ and</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lys¹⁰⁵</td>
</tr>
<tr>
<td>11</td>
<td>2074.2</td>
<td>2074.1</td>
<td>0.00</td>
<td>Glu¹²⁰⁻Glu¹²¹ + TNP</td>
<td>Lys¹⁰⁵</td>
</tr>
<tr>
<td>12</td>
<td>3038.2</td>
<td>3037.1</td>
<td>0.04</td>
<td>Glu¹⁰⁻Asp¹⁰⁶ + TNP</td>
<td>Lys¹⁰⁴⁻Lys²⁰</td>
</tr>
<tr>
<td>13</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td>Lys¹³⁰⁻Lys²⁰⁶</td>
</tr>
<tr>
<td>14</td>
<td>1061.8</td>
<td>1061.0</td>
<td>0.08</td>
<td>Leu⁴⁰⁻Asp⁴⁰ + TNP + Na</td>
<td>Lys¹³⁰⁻Lys²³³</td>
</tr>
<tr>
<td>15</td>
<td>3536.7</td>
<td>ND</td>
<td></td>
<td></td>
<td>Lys¹⁴ and</td>
</tr>
<tr>
<td>16</td>
<td>3250.0</td>
<td>3248.1</td>
<td>0.06</td>
<td>Glu¹⁰⁻Asp¹⁰⁶ + 2TNP</td>
<td>Lys¹⁴ and</td>
</tr>
</tbody>
</table>

* Fraction numbers correspond to those of fractions in Fig. 6.
* See “Results.”
* ND, not determined.

Lys²³⁶. These domains, combined with the domains accessible to NSP only in solution, may be important for the interaction of the 33-kDa protein with PSII.

**DISCUSSION**

Positive Charges Are Important for the Interaction of the 33-kDa Protein with PSII—The present results demonstrated that, while elimination of a significant number of negative charges by modification of the carboxyl groups with GME did not affect the rebinding and reactivating capabilities of the 33-kDa protein, removal of positive charges by modification of the lysyl residues with NSP or TNBS or modification of the arginyl residues with BD significantly affected the rebinding and thus the reactivating capability of the 33-kDa protein. This is largely in agreement with the finding that most of the positively charged residues are not involved in direct interaction with PSII. The other three arginyl residues, Arg⁵, Arg¹²², and Arg¹⁷₈, are not strictly conserved and may not be
important for interaction of the protein with PSII.

It should be pointed out that modification of either one or two lysyl or one or two arginyl residues did not result in a significant loss of the binding of the 33-kDa protein to PSII. This implies that the interaction of the protein with PSII involves multiple sites and that this interaction did not change much upon destruction of one or two of them. This implies that alteration of only one lysyl or arginyl residue of the 33-kDa protein may not result in a distinct phenotype in site-directed mutagenesis studies on the protein; instead, multiple lysyl or arginyl residues should be mutagenized simultaneously in order to obtain a significantly altered phenotype of the mutant strain.

**Domains of Lysyl Residues Possibly Responsible for Interaction of the Extrinsic 33-kDa Protein with PSII**—Table VI summarizes the domains determined to be accessible to NSP or TNBS only in solution, except residue Lys\(^{105}\), which was accessible to TNBS only in solution but was accessible to NHS-biotin both in solution and on PSII membranes (see also Table VI and “Results” for details). The stars indicate completely conserved residues.

![Sequence homology of the 33-kDa protein from 11 organisms currently available in data bases. The sequences are extracted from NCBI data bases. The organisms are spinach (1), pea (2), potato (3), wheat (4), Arabidopsis thaliana (5), Chlamydomonas reinhardtii (6), Euglena gracilis (7), Synechococcus sp. PCC 6301 (8), Synechocystis sp. PCC 6803 (9), Anabaena sp. PCC 7120 (10), and Synechococcus elongatus (11). Domains accessible to NSP and/or TNBS only in solution are boxed, except residue Lys\(^{14}\), which was accessible to TNBS only in solution but was accessible to NHS-biotin both in solution and on PSII membranes (see also Table VI and “Results” for details). The stars indicate completely conserved residues.](https://www.jbc.org/content/3797/7/3797.full)

**TABLE VI**

<table>
<thead>
<tr>
<th>NSP</th>
<th>TNBS</th>
<th>NHS-biotin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu(^{1})-Lys(^{4})</td>
<td>Lys(^{8})</td>
<td>Glu(^{1})-Lys(^{4})</td>
</tr>
<tr>
<td>Lys(^{6})-Lys(^{7})</td>
<td>Lys(^{6})-Lys(^{7})</td>
<td>Lys(^{20})</td>
</tr>
<tr>
<td>Lys(^{10})</td>
<td>Lys(^{10})</td>
<td>Lys(^{125})-Lys(^{136})</td>
</tr>
<tr>
<td>Lys(^{136})</td>
<td>Lys(^{136})</td>
<td>Lys(^{136})-Lys(^{136})</td>
</tr>
<tr>
<td>Lys(^{20})</td>
<td>Lys(^{20})</td>
<td>Lys(^{20})-Lys(^{20})</td>
</tr>
</tbody>
</table>

* Data from Ref. 32.

be determined unambiguously in the present study; two lysyl residues in domain Lys\(^{105}\)-Lys\(^{137}\) were modified by NSP in solution, whereas only residue Lys\(^{105}\) was modified by NSP when the 33-kDa protein was associated with PSII membranes. Apparently, there is one lysyl residue in domain Lys\(^{105}\)-Lys\(^{137}\) that is not accessible to NSP when the 33-kDa protein is bound to PSII. This residue is probably Lys\(^{130}\) as determined from another V8 protease-digested fragment, but the possibility that it is Lys\(^{105}\) cannot be excluded. In any case, the difference of modification observed between NSP and TNBS may be caused by the difference between the two modification reactions; although the molecular sizes of NSP and TNBS are not much different, modification by NSP results in the addition of a small carbon chain (N-propionyl, add-on mass 56.0 Da) whereas modification with TNBS results in the addition of the TNP group containing an aromatic ring, which is much larger in size (molecular mass 211.0 Da). When the modification was carried out on the 33-kDa protein free in solution, there was no significant difference between the number of domains modified by NSP and those modified by TNBS; there were 12 domains...
modified by NSP, whereas there were 9 modified by TNBS (not taking into consideration some peptide fragments that were not identified). When the modification was carried out on the 33-kDa protein bound to PSII, on the other hand, the domains modified by TNBS were much fewer than those modified by NSP; six domains were modified by NSP, but only one domain was modified by TNBS. This may be caused by the steric hindrance of nearby residues of either the 33-kDa protein or PSII membrane proteins against access of the large TNP group to the lysyl residues that are otherwise liable to addition of the small N-propionyl group when the modification reaction was carried out with the 33-kDa protein bound to PSII.

Based on the above considerations, it is important to use reagents with different structural and reactive groups in the modifying reactions, and the results obtained should be combined in order to get a more complete understanding on the possibly important residues. We therefore consider that the domains modified either by NSP or TNBS only when the 33-kDa protein is in solution are all shielded from contact with bulk solution upon binding to PSII and may be important for the interaction of the protein with PSII. These are Lys4, Lys14, Lys20, Lys66–Lys76, Lys101, Lys105, Lys130, Lys159, Lys186, and Lys230–Lys236. These domains included five of the seven completely conserved lysyl residues present in 11 sequences of the 33-kDa protein from cyanobacteria to higher plants (Fig. 7): Lys66, Lys76, Lys130, Lys159, and Lys236. In addition, the residue Lys186 is completely conserved among eight organisms and is changed to Arg with a similar positive charge only in three cyanobacteria (Fig. 7). The present results thus strongly suggest that these conserved lysyl residues are important for binding of the 33-kDa protein to PSII membranes. The other two conserved lysyl residues that are not unambiguously identified as accessible to modification only in solution are Lys40 and Lys180, whether these two lysyl residues are important for interaction of the protein with PSII is not clear at present.

The domains determined above included the four domains that are accessible to NHS-biotin only when the 33-kDa protein is free in solution, as determined previously by Frankel and Bricker (32): Glu1–Lys4, Lys51, Lys101–Lys105, and Lys159–Lys186 (Table VI). Among these four domains, domains Glu1–Lys4 and Lys159–Lys186 were identified as being modified by both NSP and TNBS only when the 33-kDa protein is in solution. Lys20 and Lys101–Lys105 were modified by TNBS only in solution but not by NSP (the domain Lys101–Lys105 may be modified by NSP only in solution as mentioned above, but our present results did not allow us to draw an unambiguous conclusion). The other domains that were accessible to NSP or TNBS but not to NHS-biotin only in solution are Lys40–K (TNBS), Lys51, Lys65–Lys76, and Lys200–Lys236 (NSP and TNBS). Among these domains, only Lys40 was accessible to NHS-biotin both in solution and on PSII membranes (32), suggesting that this residue may not directly interact with PSII. The other domains were either completely inaccessible to NHS-biotin (Lys130) or less accessible to NHS-biotin than to the modifiers used in the present work (Lys66–Lys76, Lys200–Lys236). These differences may be attributable to the differences among, in addition to the modifying reactions as described above, the properties of the three modifying reagents used; NSP and TNBS are hydrophilic reagents and may be more efficient in modifying residues located in the hydrophilic part of the protein, whereas NHS-biotin is largely hydrophobic and therefore is expected to access residues in the hydrophobic part of the protein. In any events, our present studies confirmed and further extended the previous results of Frankel and Bricker as to the domains that are important for the interaction of the 33-kDa protein with PSII. These results should provide important clues to further site-directed mutagenesis studies on the 33-kDa protein.

In conclusion, the present results demonstrated that, while negative charges of carboxylic residues (and the C terminus) of the 33-kDa protein do not participate in the functional binding of the protein to PSII, positive charges of lysyl and arginyl residues are important for its binding to, and thus function in, PSII. The domains containing lysyl residues determined to be exposed to bulk solution only when the 33-kDa protein is free in solution are Lys4, Lys14, Lys66–Lys76, Lys101, Lys105, Lys180, Lys186, and Lys230–Lys236. These domains may directly interact with PSII intrinsic components.
Identification of Domains on the Extrinsic 33-kDa Protein Possibly Involved in Electrostatic Interaction with Photosystem II Complex by Means of Chemical Modification

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