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Association of the 17-kDa Extrinsic Protein with Photosystem II in Higher Plants†‡

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ABSTRACT: The structural association of the spinach 17-kDa extrinsic protein of photosystem II with other extrinsic and membrane-bound components of the photosystem was investigated by labeling the 17-kDa extrinsic protein with the amino-group-specific reagent N-hydroxysuccinimidobiotin both on intact photosystem II membranes or as a free protein in solution. After isolation of the biotinylated molecules, the modified 17-kDa proteins were allowed to rebind to photosystem II membranes which were depleted of the 17-kDa component. Differential binding of the protein biotinylated in solution compared to unmodified 17-kDa protein or 17-kDa protein modified on PS II membranes was observed. This indicated possible steric or ionic interference because of biotinylated lysyl residues present on the protein modified in solution. Biotinylated sites on the different modified 17-kDa proteins were identified by trypsin and Staphylococcus V8 protease digestion, followed by affinity chromatography enrichment of the biotinylated peptides and analysis of the peptide fragment mixture by nanospray liquid chromatography—tandem mass spectrometry. Four lysyl residues that were modified when the protein was biotinylated in solution were not biotinylated when the protein was modified on the PS II membrane (90 K, 96 K, 101 K, and 102 K). These residues appear to identify a protein domain involved in the interaction of the 17-kDa protein with the other components of the photosystem.

Photosystem II (PS II) is composed of both intrinsic and extrinsic protein components, which participate in the photoenergetically driven oxidation/reduction reaction of photosynthesis. Associated with this oxygen-evolving complex in higher plants are three extrinsic membrane proteins, with molecular masses of 33, 24, and 17 kDa, that are encoded by the psbO, psbP, and psbQ genes, respectively. The 24-, and 17-kDa proteins can be removed by treatment with 1.0 M NaCl. This results in a dramatically lower oxygen-evolving ability of PS II membranes. Recovery of this lost oxygen evolution capability can be accomplished via reconstitution with the 24- and 17-kDa protein or through the addition of calcium and chloride ions. Removal of the 33-kDa protein requires a high concentration of alkaline Tris, CaCl₂, or NaCl—urea. While the loss of the two smaller extrinsic components (24 and 17 kDa) leads to a significant loss of oxygen evolution that can be compensated for with calcium and chloride ions, the 33-kDa extrinsic protein is required for the high rates of oxygen evolution in vivo and in isolated PS II preparations.

In higher plants, the presence of the 33-kDa component is required for binding of the 24-kDa protein. Similarly, the presence of the 24-kDa component is needed for the 17-kDa protein to bind to the complex. The high-resolution crystal structures of the 17-kDa protein exhibit a compact 4-helix bundle core with an N-terminal domain which was not fully resolved in the crystal structure (Figure 1A). The N-terminal 12 amino acid residues had earlier been identified as being necessary for the binding of the 17-kDa protein to PS II. The loss of these residues led to a marked decrease in the ability of this component to bind to the photosystem. However, it is unclear if the N terminus of the 17-kDa protein is the only determinant responsible for the binding of this component to the photosystem.

Salt-washing with 1.0 M NaCl, in addition to removing the 24- and 17-kDa components, also releases a prolyl endoprotease specific for the proline-rich N-terminal segment of the 17-kDa protein. Lowering the salt concentration through subsequent dialysis appears to activate the protease, cleaving off the N-terminal 12 residues of the 17-kDa protein. A novel method of removing the 17-kDa protein without the concerted removal of the 24-kDa protein and which prevents digestion of the 17-kDa protein by the prolyl endoprotease is employed in this paper.
are not labeled when the 17-kDa protein is associated with the PS II membrane. These residues appear to define a site of interaction between the 17-kDa protein and PS II.

**MATERIALS AND METHODS**

**Isolation of PS II Membranes and the 17-kDa Protein.** Chloroplasts were isolated from spinach bought at a local market (16). The Chl concentration was measured by the method of Arnon (17). Oxygen-evolving PS II membranes were prepared by the method of Berthold et al. (18), with the modifications described by Ghanotakis and Babcock (19). Typical preparations had a Chl/Chl ratio of 1.9–2.0.

The 17-kDa protein was removed (along with the MSP) from PS II membranes by extraction with 40% MeOH and 1.0 M NaCl, in 50 mM Tricine-NaOH at pH 9.0. The presence of 40% MeOH minimizes the removal of the 24-kDa protein, while the high pH and salinity suppress the activity of the released prolyl endopeptidase. PS II membranes were incubated for 10 min at 4 °C, followed by centrifugation at 30000 g for 20 min. The supernatant was collected and dialyzed against a 100:1 volume of deionized water. After dialysis, the extract was concentrated to 1–2 mL with a centrifugal ultrafiltration device (Centricron 20, 10-kDa cutoff, Polysciences, Inc.). The concentrate was then loaded onto a CM-Toyopearl 650 M chromatography column and eluted with a 0–250 mM NaCl gradient in 20 mM Na-KPO4 at pH 6.5. The MSP eluted in the void volume of the column along with a small amount of contaminating 24-kDa protein, while the 17-kDa protein eluted at about 100 mM NaCl (data not shown).

Analytical PAGE of the PS II proteins was performed under conditions described by Delepelaire and Chua (20) in gradient 12.5–20% polyacrylamide gels. The resolved proteins were electroblotted onto PVDF membranes (Immobilon-P, Millipore Co.). After blocking for 2 h with 5% nonfat dry milk in 150 mM NaCl and 10 mM Tris-HCl at pH 7.4, the blots were washed extensively with 150 mM NaCl and 10 mM Tris-HCl at pH 7.4. For immunological detection, a monoclonal antibody, FCC4 (21), was used as the primary antibody and an anti-mouse IgG-peroxidase conjugate was used as a secondary antibody. 4-Chloro-1-naphthol and H2O2 were used to visualize the peroxidase-labeled bands.

**Modification of the 17-kDa Protein with NHS–Biotin.** For biotinylation of the 17-kDa protein while it is associated with PS II, PS II membranes were centrifuged and resuspended in 300 mM sucrose, 10 mM MgCl2, 15 mM NaCl, and 50 mM Tes-NaOH at pH 7.0 at 1.0 mg of Chl/mL and kept at 0–4 °C throughout the procedure. Exposed amino groups (lysyl residues and the N terminus) on the 17-kDa protein were labeled with NHS–biotin as described (22), with the modifications that the NHS–biotin concentration during labeling was 50 μM and the labeling time was 1.5 h. The labeling reaction was stopped by the addition of Tris-HCl at pH 6.8, to a concentration of 50 mM. The residual NHS–biotin was removed by washing the membranes twice with 300 mM sucrose, 10 mM MgCl2, 15 mM NaCl, and 50 mM Mes-NaOH at pH 6.0 (resuspension buffer). The biotinylated 17-kDa protein was then purified as described above.

Labeling of the purified 17-kDa extrinsic protein in solution was performed in a similar manner at a protein

**FIGURE 1: Structure of the 17-kDa protein (PDB 1VYK).** (A) Cartoon diagram showing the 4-helix bundle and the relatively unstructured N-terminal domain. Note that residues 36S–33Y are not resolved in the current structure. The four α-helixes are labeled I–IV. A total of 14 lysyl residues are present in the protein; these are shown in a space-filling representation superimposed upon the cartoon diagram. All 14 of these lysyl residues are labeled when the protein is modified in solution. (B) Four lysyl residues, which are not accessible to the modification reagent when the protein is associated with the PS II membrane, are shown in magenta with their residue numbers. In this view, helix IV is partially obscured and is not labeled.

In this study, NHS–biotin was used to modify the 17-kDa extrinsic protein both in association with the PS II membranes and as a free protein in solution. Rebinding experiments indicated that the unmodified 17-kDa protein and 17-kDa protein that was modified while associated with PS II rebound equivalently, while the 17-kDa protein that was modified in solution exhibited defective rebinding kinetics. To map the location of the residues modified by NHS–biotin treatment, the different biotinylated 17-kDa proteins were isolated and digested with either trypsin or Staphylococcus V8 protease; the peptide fragments were isolated by affinity chromatography and were then analyzed by nanospray LC-tandem mass spectroscopy. Four lysyl residues (99K, 96K, 101K, and 102K) are labeled with NHS–biotin when the 17-kDa protein is modified in solution but
concentration of 138 µg/mL in 300 mM sucrose, 10 mM MgCl₂, 15 mM NaCl, and 50 mM Tes-NaOH at pH 7.0. The labeling reaction was stopped by the addition of Tris-HCl at pH 6.8, to a concentration of 50 mM, and the residual NHS–biotin was removed by centrifugal ultrafiltration in a Centricon 20 device with a 10-kDa cut off (Polysciences, Inc.). The purified proteins were quantified using an extinction coefficient of 12 mM⁻¹ cm⁻¹ at 277 nm (23).

Rebinding of the Modified 17-kDa Proteins to PS II Membranes. The rebinding of unmodified and modified versions of the 17-kDa protein to PS II membranes, which were depleted of only the 17-kDa protein, was performed. Depleted membranes were prepared by incubating PS II membranes with 20% MeOH, 100 mM NaCl, and 50 mM Mes-NaOH at pH 6.0 (24) for 1 h. After centrifugation to recover the 17-kDa protein-depleted membranes, they were washed twice with and resuspended in resuspension buffer. Varying amounts of the different 17-kDa protein preparations were incubated with 17-kDa protein-depleted membranes. After incubation for 30 min, the 17-kDa protein-reconstituted membranes were recovered by centrifugation at 14000g for 5 min and washed twice with resuspension buffer. After centrifugation, the membrane pellet was resuspended in resuspension buffer and the Chl concentration was determined. A total of 15 µg of Chl was loaded per lane for PAGE. The gels were electroblotted onto PVDF membranes and probed with the FCC4 antibody as described above. After drying, the “Western” blots were scanned (300 dpi resolution, 256 gray-scale levels), and the integrated optical densities of the 17-kDa bands were determined using SigmaGel version 1.0 (Jandel Scientific, Inc.). The bound amounts of 17-kDa protein were normalized to the amount present in unwashed PS II membranes. The PS II concentration was estimated assuming 260 Chl/PS II (25).

Protease Digestion and Purification of Biotinylated Peptides. For mass spectrometry, a sample of each biotinylated protein was brought to 10% TCA and the protein precipitate was collected by centrifugation, then washed twice with cold 100% acetone, dried under vacuum, and dissolved in 8 M urea and 400 mM ammonium bicarbonate (26). The solubilized protein was then diluted to 2.0 M urea and 100 mM ammonium bicarbonate and digested overnight at 37 °C with either trypsin or *Staphylococcus* V8 protease. The final 17-kDa protein/protease ratio was 25:1. The digestion was stopped by the addition of a protease inhibitor cocktail (Sigma Chemical Co.) prior to purification of the biotinylated peptides on a SoftLink Resin monomeric avidin column (Promega), which was prepared according to the instructions of the manufacturer.

The peptide sample was loaded on the avidin column, and the column was washed with 5 column volumes of 100 mM NH₄HCO₃ at pH 7.0. The bound biotinylated molecules were then eluted with 10% acetic acid. The eluent fractions were then eluted with 10% acetic acid. The eluent fractions were subjected to liquid chromatography–tandem mass spectrometry analysis, a Waters CapLC coupled to a Q-TOF II mass spectrometer was used. The samples were injected onto a 75 µm i.d. × 10 cm spraying capillary packed with 5 µm C₁₈ beads. The flow rate was set to 7 µL/min split to approximately 200 nL/min before reaching the column. A 75 min gradient was used to obtain good peptide separation. Buffer A consisted of 95% water, 5% acetonitrile, and 0.1% formic acid, and buffer B was 95% acetonitrile, 5% water, and 0.1% formic acid. Mass spectrometry results were analyzed with the GPMAW software package (Lighthouse Data, Sweden) and the MASCOT software suite (Matrix Science, Ltd.). PYMOL was used for production of molecular graphics (http://pymol.sourceforge.net/).

RESULTS AND DISCUSSION

The ability of the NHS–biotin-modified proteins to bind to PS II membranes is shown in Figure 2. The 17-kDa protein-depleted PS II membranes were incubated with various amounts of either unmodified or NHS–biotin-modified 17-kDa proteins. The membranes were washed extensively, and the amount of bound 17-kDa protein was analyzed by PAGE followed by “Western” blotting, probing with the FCC4 monoclonal antibody, color development, and image analysis. The unmodified 17-kDa protein bound freely to PS II membranes, with saturation appearing to occur at about 1 mole of 17-kDa protein/mole of PS II. The binding of 17-kDa protein which had been modified with NHS–biotin while associated with the PS II membranes was indistinguishable from that observed with the unmodified protein. This indicated that the lysyl residues labeled when the 17-kDa protein was modified on the membrane did not inhibit rebinding. Reconstitution with the 17-kDa protein that had been modified in solution, however, yielded a radically different binding curve. Binding of this protein was markedly inhibited and exhibited only a minor increase throughout the range of protein ratios examined during the reconstitution. This result indicates that modification of the lysyl residues exposed when the 17-kDa component was labeled in solution dramatically alters the ability of the modified protein to bind to PS II. The binding that was observed with this protein preparation may be the result of a nonspecific interaction.

![Figure 2: Reconstitution of 17-kDa protein-depleted PS II membranes with control and modified 17-kDa proteins.](image)
with the PS II membrane. Similar binding kinetics were observed earlier in studies that documented the necessity of the N terminus of this protein for its binding to PS II (14). In an effort to understand which domains on the 17-kDa protein are interacting with PS II, we have identified the regions of this component that were differentially labeled with NHS–biotin. Tables 1 and 2 list the peptide assignments obtained by digestion of the 17-kDa protein that had been labeled on and off of the membrane, respectively.

In these studies, two proteases, trypsin or Staphylococcus V8 protease, were employed. The use of different proteases, which differentially cleave the 17-kDa protein, increases the number of peptides that can be analyzed in these experiments. Increasing the number of peptides that can be analyzed increases the probability that a representative and more complete sample of proteolytic fragments will be obtained for analysis.

Overnight digestion of the modified 17-kDa proteins with either trypsin or Staphylococcus V8 protease yielded a mixture of completely and partially digested proteolytic fragments. Such peptide mixtures can be analyzed with high mass accuracy by nanospray LC–tandem mass spectrometry. Modification with NHS–biotin leads to the addition of 226.08 Da/modified lysyl residue. The amino acid sequence information from the analysis of collision-induced daughter ions allows the identification of the specific NHS–biotin-modified lysyl residues. The Mascot Program suite (http://www.matrixscience.com) and GPMAW version 6.0 (Lighthouse Data) were used to facilitate peptide assignments.

For the protein that was modified while associated with the PS II membrane, 11 biotinylated tryptic peptides and 4 biotinylated V8 protease peptides were identified. In addition, a number of nonbiotinylated peptides were observed (data not shown). These results highlight the fact that, while the monomeric avidin column enriches the protein samples for biotinylated peptides, some nonbiotinylated peptides are retained by the matrix of the column and are not fully

Table 1: Assignments of Peptides Observed after Trypsin or Staphylococcus V8 Protease Digestion of 17-kDa Protein Labeled on the Photosystem II Membrane with NHS–Biotin

<table>
<thead>
<tr>
<th>observed mass (ppm)</th>
<th>peptide assignment</th>
<th>modified lysyl residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1390.58</td>
<td>28D−3H + 1 biotin</td>
<td>35K</td>
</tr>
<tr>
<td>1440.72</td>
<td>901−102K + 1 biotin</td>
<td>9K or 98K</td>
</tr>
<tr>
<td>1483.79</td>
<td>52A−63K + 1 biotin</td>
<td>8K</td>
</tr>
<tr>
<td>1552.70</td>
<td>69K−79R + 1 biotin</td>
<td>69K</td>
</tr>
<tr>
<td>1896.92</td>
<td>11L−125K + 1 biotin</td>
<td>125K</td>
</tr>
<tr>
<td>1944.01</td>
<td>54V−68R + 1 biotin</td>
<td>68R</td>
</tr>
<tr>
<td>2072.09</td>
<td>54V−49K + 1 biotin</td>
<td>49K</td>
</tr>
<tr>
<td>2093.96</td>
<td>131Y−149G + 1 biotin</td>
<td>149G</td>
</tr>
<tr>
<td>2452.24</td>
<td>105S−123K + 1 biotin</td>
<td>123K</td>
</tr>
<tr>
<td>2666.30</td>
<td>125S−147K + 1 biotin</td>
<td>147K</td>
</tr>
<tr>
<td>2670.38</td>
<td>10K−123K + 1 biotin</td>
<td>123K</td>
</tr>
<tr>
<td>2907.47</td>
<td>124L−147K + 1 biotin</td>
<td>147K</td>
</tr>
<tr>
<td>1640.69</td>
<td>13K−143E + 1 biotin</td>
<td>143E</td>
</tr>
<tr>
<td>2684.32</td>
<td>37R−58E + 1 biotin</td>
<td>58E</td>
</tr>
<tr>
<td>2940.28</td>
<td>107L−131E + 1 biotin</td>
<td>131E</td>
</tr>
<tr>
<td>2940.42</td>
<td>10L−131E + 1 biotin</td>
<td>131E</td>
</tr>
</tbody>
</table>

- Predicted mass determined from the amino acid sequence of the mature protein. * Monoisotopic mass. c Labeled lysyl residue, ambiguity resolved by MS/MS.

The 17-kDa protein contains 14 lysyl residues (Figure 1), 10 of which were observed to be biotinylated when the protein was modified while associated with PS II (Table 1). These residues, 5K, 53K, 63K, 99K, 99K, 110K, 123K, 123K, and 147K, are consequently exposed to the bulk solvent and the labeling reagent. We have taken a very conservative approach in identifying the labeled residues. All peptide assignments are based on tandem mass spectrometry sequence information, which identifies the specifically modified lysyl residue(s). It should be noted that Table 1 contains two V8 protease peptides that were observed at a nominal mass of 2940 Da. Both of these were assigned to the peptide 107L−131E, which contained one biotin modification. This peptide contains three possible biotinylatable lysyl residues, 110K, 123K, and 147K. Analysis of the tandem mass spectrometry data indicated that one of these peptides contained a biotinylated 123K and the other contained a biotinylated 110K.

Table 2 lists the peptide assignments made for peptides generated by proteolytic digestion of the 17-kDa protein that had been biotinylated in solution. The 14 biotinylated tryptic
peptides and 7 biotinylated V8 protease peptides were observed. All 14 lysyl residues which are present in the 17-kDa protein (\(^{55}\)K, \(^{53}\)K, \(^{63}\)K, \(^{69}\)K, \(^{90}\)K, \(^{96}\)K, \(^{101}\)K, \(^{102}\)K, \(^{110}\)K, \(^{123}\)K, \(^{125}\)K, \(^{132}\)K, and \(^{137}\)K) were identified as modified on the 17-kDa protein that was biotinylated in solution. These results indicate that, in solution, all lysyl residues present in the protein are exposed at the protein surface and are accessible to the bulk solvent and the labeling reagent.

Consequently, four residues (\(^{96}\)K, \(^{90}\)K, \(^{101}\)K, and \(^{102}\)K) are labeled only in solution but are not labeled when the 17-kDa protein is associated with PS II. The locations of these residues are shown in Figure 1B. All of these residues cluster in a domain that bridges the end of helix II and the start of helix III. The NHS–biotinylation of one or more of these residues is responsible for the loss of normal binding of the 17-kDa protein to PS II (Figure 2). We hypothesize that these clustered residues are oriented to face the PS II complex (Figure 1B). A number of hypotheses could account for this observation. First, it is possible that the modified lysyl residues are directly involved in the formation of charge-pair interactions with the other components in PS II. While such charge-pair interactions were not observed between the 17-kDa protein and other PS II components using EDC cross-linking (a zero-length cross-linker, Bricker and Frankel, unpublished observations), it should be noted that if the charge pair is buried at the face of the interaction (and consequently not accessible to the cross-linking reagent) it would not be identified by this technique. A second possibility is that the labeling with NHS–biotin sterically interferes with the association of the 17-kDa protein to PS II. The presence of the biotinylation moiety may prevent the formation of other protein–protein interactions that are required for effective binding of the 17-kDa protein to PS II. It is impossible at this point in time to differentiate these (and possibly other) mechanisms.

The N-terminal 12 amino acid residues of the protein had previously been shown to be necessary for the 17-kDa protein to bind to PS II (14). It was unclear, however, if this domain was also sufficient for the interaction of the protein with the photosystem. Our results indicate that other domains on the 17-kDa protein may also be necessary for normal binding and interaction with the photosystem.

Which other components of PS II could be interacting with the 17-kDa protein? The 24-kDa protein is a prime candidate. It has been previously shown that the 24-kDa protein is required for 17-kDa protein binding (30, 31). Additionally, unidentified residues on the 24-kDa protein are within 11 Å of residues on the 17-kDa protein, because these two components can be cross-linked with homobifunctional cross-linking reagents that span 11 Å (32). The 33-kDa protein has also been implicated in providing binding determinants for the 17-kDa protein. In cross-reconstitution experiments, extrinsic protein-depleted PS II membranes, which had been reconstituted with either cyanobacterial or red algal 33-kDa proteins, could only partially rebind spinach 24-kDa protein and could not bind spinach 17-kDa protein. This indicates that there may exist structural determinants on the higher plant 33-kDa protein which are required for the binding of the 24- and 17-kDa proteins and which are absent in the homologous cyanobacterial and red algal 33-kDa proteins (33). Finally, it is possible that the 17-kDa protein interacts directly with unidentified intrinsic membrane protein components of PS II. In *Chlamydomonas*, the 17-kDa protein can bind to its functional site on the photosystem independently of the other extrinsic proteins (34). While this ability is not observed in higher plants, it does highlight the possibility that intrinsic PS II components may interact directly with the extrinsic 17-kDa protein.

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