Intramolecular Cross-linking of the Extrinsic 33-kDa Protein Leads to Loss of Oxygen Evolution but Not Its Ability of Binding to Photosystem II and Stabilization of the Manganese Cluster*

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The extrinsic 33-kDa protein of photosystem II (PSII) was intramolecularly cross-linked by a zero-length cross-linker, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The resulting cross-linked 33-kDa protein rebound to urea/NaCl-washed PSII membranes, which stabilized the binding of manganese as effectively as the untreated 33-kDa protein. In contrast, the oxygen evolution was not restored by binding of the cross-linked protein, indicating that the binding and manganese-stabilizing capabilities of the 33-kDa protein are retained but its reactivating ability is lost by intramolecular cross-linking of the protein. From measurements of CD spectra at high temperatures, the secondary structure of the intramolecularly cross-linked 33-kDa protein was found to be stabilized against heat treatment at temperatures 20 °C higher than that of the untreated 33-kDa protein, suggesting that structural flexibility of the 33-kDa protein was much decreased by the intramolecular cross-linking. The rigid structure is possibly responsible for the loss of the reactivating ability of the 33-kDa protein, which implies that binding of the 33-kDa protein to PSII is accompanied by a conformational change essential for the reactivation of oxygen evolution. Peptide mapping, N-terminal sequencing, and mass spectroscopic analysis of protease-digested products of the intramolecularly cross-linked 33-kDa protein revealed that cross-linkings occurred between the amino group of Lys48 and the carboxyl group of Glu46, and between the carboxyl group of Glu86 and the amino group of Lys14. These cross-linked amino acid residues are thus closely associated with each other through electrostatic interactions.

PSII† is a multisubunit pigment-protein complex which catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. The minimum unit for PSII capable of oxygen evolution under physiological conditions contains seven major intrinsic proteins of the reaction center peptides D1 and D2, two apoproteins of cytochrome b559, psbI gene product, and two chlorophyll-binding peptides CP47 and CP43, and three extrinsic proteins of 33, 23, and 17 kDa which are associated with the luminal surface of thylakoid membranes (1–3). The extrinsic 23- and 17-kDa proteins play a role in regulating the PSII affinity for calcium and chloride, and can be removed by treatment with 1.0–2.0 M NaCl (4–9). In cyanobacterial and red algal PSII, these two extrinsic proteins are absent, but a low-potential cytochrome c550 and a 12-kDa protein have been found as the alternative extrinsic components (10–12).

The extrinsic 33-kDa protein, on the other hand, is present in all oxygenic photosynthetic organisms from cyanobacteria to higher plants and plays an important role in stabilizing binding and maintaining functional conformation of the manganese cluster which directly catalyzes the H2O-splitting reaction (for reviews, see Refs. 13–15). Removal of the 33-kDa protein from PSII membranes by washing with high concentration of divalent cations (16) and urea plus NaCl (17) significantly decreases the oxygen evolving activity but the activity can be considerably restored by rebinding of the protein (17–19). Removal of the protein also leads to a gradual liberation of two out of the four manganese per PSII (20). Immobilization of the protein with PSII intrinsic components by a water-soluble carbodiimide, EDC, 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 and the oxygen evolving activity against these treatments (21–23). 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 (21, 24–26). Recently, we showed that positive charges on the 33-kDa protein are important for the electrostatic interaction with PSII intrinsic proteins, whereas negative charges on the protein do not contribute to such interaction (27). Furthermore, we proposed that the positive charges of ε-amino groups on Lys4, Lys20, Lys46 or Lys76, Lys101, Lys105, Lys130, Lys152, Lys186, and one or two Lys in Lys210–Lys236 in the extrinsic 33-kDa protein

high performance liquid chromatography; Mes, 4-morpholinethanesulfonic acid.
electrostatically interact with negative charges on PSII intrinsic proteins (27).

Reconstitution experiments have indicated that the three extrinsic proteins bind to the PSII complex in the order of the 33-, 23-, and 17-kDa proteins (28–30). Among these three extrinsic proteins, the 33-kDa protein is required for stoichiometric and functional binding of the 23-kDa protein, whereas both the 33- and 23-kDa proteins are required for functional binding of the 17-kDa protein. The extrinsic proteins cannot, however, directly bind to each other when they are not associated with the PSII complex (24). These results suggest that binding of the extrinsic proteins to the PSII complex alters the conformation of the extrinsic proteins themselves and/or that of the intrinsic part of the complex so as to create the binding sites for the other extrinsic proteins (24).

To examine whether conformational changes occur with the 33-kDa protein accompanying its binding to PSII and the possible importance of such conformational changes, we performed intramolecular cross-linking of the 33-kDa protein with a water-soluble carbodiimide, EDC, by which the conformational changes of the protein are expected to be suppressed. The intramolecularly cross-linked 33-kDa protein was found to retain the rebinding and manganese-stabilizing capabilities but not the reactivating ability, suggesting that a suitable flexibility of the 33-kDa protein is needed for its full functioning in oxygen evolution.

MATERIALS AND METHODS
Preparation and Cross-linking—Oxygen-evolving PSII membranes were prepared from spinach chloroplasts with Triton X-100 as in Ref. 31, with slight modifications as described in Ref. 21. The PSII membranes were suspended in medium A containing 40 mM Mes (pH 6.5), 0.4 mM succrose, 10 mM NaCl, and 5 mM MgCl2 and stored in liquid nitrogen until use. The extrinsic 33-kDa protein was extracted from the PSII membranes by 1 M CaCl2 treatment (16) and purified according to Refs. 27 and 32. For cross-linking, the purified 33-kDa protein was passed through a Sephadex G-25 column equilibrated with distilled water. The concentration of the 33-kDa protein was determined using an extinction coefficient of 16 mM

\[ \text{dimer} \]

\[ \text{Monomer} \]

FIG. 1. SDS-polyacrylamide gel electrophoresis of EDC-treated 33-kDa protein. The purified 33-kDa protein was treated with EDC as described under “Materials and Methods” (lane 1), and then passed through a Sephacryl S-100HR column, by which two proteins peaks were separated. The first peak (lane 2) corresponded to a dimer and the second peak (lane 3) to a monomer.

0 °C in the dark were centrifuged at 35,000 \times g for 10 min and manganese remaining in the supernatant was assayed with a Hitachi polarized Zeeman atomic absorption spectrophotometer (Z-8000). Amounts of manganese per PSII were estimated by assuming the antenna size of PSII as 250 Chl. The circular dichroism (CD) measurements of the native or intramolecularly cross-linked 33-kDa protein were performed with a JASCO J-800A spectropolarimeter as described in Ref. 36.

Protease Digestion, HPLC Separation, Mass Spectroscopic, and N-terminal Sequencing Analysis—The untreated and intramolecularly cross-linked 33-kDa proteins were denatured in 8 M urea and 25 mM Mes (pH 6.5) at 37 °C for 15 h, and then 3 M Tris-HCl (pH 8.5) was added to a final concentration of 2 M urea. The denatured 33-kDa protein was digested first with lysyl endopeptidase at a protein to lysyl endopeptidase ratio of 50 (w/w) for 15 h at 37 °C, and then with another lysyl endopeptidase at the same concentration for 15 h at 37 °C. The digested mixture was subjected to a reverse phase column (Bondsphere 5 Å, C4 300A, 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. Each fraction was collected, dried, and resolubilized in 2 μl of 67% acetic acid out of which, 1 μl was mixed with a same volume of matrix (a mixture of 1:1 volume 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 protein sequence. The peptides obtained from HPLC were also analyzed for their N-terminal sequence by Edman degradation of the peptides followed by sequence analysis with a protein sequencer (Applied Biosystem, model 477A and 476A).

RESULTS
Preparation of Intramolecularly Cross-linked 33-kDa Protein—To suppress the formation of intermolecularly cross-linked products, a diluted solution of the 33-kDa protein (4 μg/ml) was used for treatment of EDC. A small amount of intermolecularly cross-linked product was, however, formed as shown in Fig. 1. The polypeptide pattern of the EDC-treated 33-kDa protein showed two Coomassie Brilliant Blue-stained bands with apparent molecular masses of about 55 and 27 kDa (lane 1), which correspond to dimer and monomer of the 33-kDa protein resulting from intermolecular and intramolecular cross-linking, respectively. No trimer and polymer are formed under the conditions employed here. The intramolecularly (dimer) and intramolecularly (monomer) cross-linked products of the 33-kDa protein could be clearly separated by a Sephacryl S-100HR column (lanes 2 and 3); upon electrophoresis, the intramolecularly cross-linked 33-kDa protein migrated faster and appeared as a broader band as compared with the untreated protein.

Reconstitution of the Intramolecularly Cross-linked 33-kDa Protein to PSII—The three extrinsic proteins of 33, 23, and 17...
kDa were removed by washing PSII with 2.6 M urea plus 0.2 M NaCl (lanes 1, 2, Fig. 2) (17). The native 33-kDa protein was able to rebind to urea/NaCl-washed PSII completely (lane 3). Because the intramolecularly cross-linked 33-kDa protein migrated faster than the native 33-kDa protein and thus comigrated with the large amount of LHC II bands, detection of the 33-kDa protein reconstituted was done as follows. PSII membranes reconstituted with the native or intramolecularly cross-linked 33-kDa protein were again treated with 2.6 M urea plus 0.2 M NaCl and centrifuged at 35,000 g for 10 min. The supernatants were concentrated 5-fold by ultrafiltration as the intramolecularly cross-linked 33-kDa protein showed a much weaker Coomassie Brilliant Blue staining intensity than the native 33-kDa protein. The result shown in lane 4R of Fig. 2 indicates that the intramolecularly cross-linked 33-kDa protein reconstituted to PSII to a significant amount. To estimate the amount of the intramolecularly cross-linked 33-kDa protein reconstituted, the intramolecularly cross-linked 33-kDa protein corresponding to the amount when the protein was completely rebound was electrophoresed together (lanes 3C and 4C). The comparison of Coomassie Brilliant Blue staining intensities in lanes 4C and 4R revealed that the intramolecularly cross-linked 33-kDa protein was completely rebound to PSII. It should be noted that the band of the intramolecularly cross-linked 33-kDa protein was completely rebound to PSII irrespective of the heterogeneous cross-linking. These results indicate that the binding sites of the 33-kDa protein remain intact after intramolecular cross-linking.

Table I shows oxygen-evolving activity of urea/NaCl-washed PSII membranes reconstituted with the native or intramolecularly cross-linked 33-kDa protein. Removal of the three extrinsic proteins by urea/NaCl wash reduced the oxygen evolving activity to 4% of the original activity. The activity was restored to 66% by reconstitution with the native 33-kDa protein, whereas it was scarcely restored by reconstitution with the intramolecularly cross-linked 33-kDa protein. This indicates that the intramolecularly cross-linked 33-kDa protein lost its reactivating ability of oxygen evolution even though it was completely rebound to PSII.

When urea/NaCl-washed PSII membranes lacking the three extrinsic proteins were incubated at 0 °C in the dark, two out of the four manganese per PSII released after 48 h (Fig. 3) (20). Reconstitution of the native 33-kDa protein effectively suppressed the release of manganese (Fig. 3). Reconstitution of the intramolecularly cross-linked 33-kDa protein also suppressed the manganese release equally effectively (Fig. 3). These results indicate that the cross-linked 33-kDa protein still retained its ability to stabilize binding of the manganese cluster.

**Table I**

| Reconstitution of the native or intramolecularly cross-linked 33-kDa protein with urea/NaCl-washed PSII membranes | Oxygen evolution | Reactivation |
|---------------------------------------------------------------|-----------------|-------------|
| PS II membrane                                              | 534             | %           |
| Urea/NaCl-washed PS II membrane                              | 22              | 100         |
| + Native 33-kDa protein                                      | 351             | 36          |
| + Intramolecularly cross-linked 33-kDa protein               |                 | 4           |

**FIG. 2.** Reconstitution of the native or intramolecularly cross-linked 33-kDa protein with urea/NaCl-washed PSII membranes. Lane 1, control PSII membranes (0.02 mg of Chl); lane 2, urea/NaCl-washed PSII membranes (0.02 mg of Chl); lane 3, urea/NaCl-washed PSII membranes reconstituted with the native 33-kDa protein (0.02 mg of Chl); lane 4, urea/NaCl-washed PSII membranes reconstituted with the intramolecularly cross-linked 33-kDa protein (0.02 mg of Chl); lane 3C, the native 33-kDa protein (0.4 nmol); lane 3R, the native 33-kDa protein extracted from urea/NaCl-washed PSII membranes (0.1 mg of Chl, corresponding to about 0.4 nmol of PSII reaction center assuming there is 250 Chl/PSII) which had been reconstituted with the native 33-kDa protein; lane 4C, the intramolecularly cross-linked 33-kDa protein (0.4 nmol); lane 4R, the intramolecularly cross-linked 33-kDa protein extracted from urea/NaCl-washed PSII membranes (0.1 mg of Chl) which had been reconstituted with the intramolecularly cross-linked 33-kDa protein.

**FIG. 3.** Stabilization of manganese binding by reconstitution of the native or intramolecularly cross-linked 33-kDa protein with urea/NaCl-washed PSII membranes. The urea/NaCl-washed PSII before or after reconstitution with the native or intramolecularly cross-linked 33-kDa protein was incubated at 0 °C in the dark, and its manganese content bound was determined at the designated time points.
intramolecularly cross-linked 33-kDa protein appeared at 90 °C (Fig. 4B). These results indicate that structural flexibility of the 33-kDa protein was significantly decreased by intramolecular cross-linking.

Identification of the Intramolecular Cross-linking Sites—Fig. 5 shows the peptide maps of the native (A) and the intramolecularly cross-linked 33-kDa protein (B) which had been digested with lysyl endopeptidase and separated by reversed-phase HPLC. Peptide peaks 2, 3, 4, and 6 drastically decreased and new peptide peaks A and B appeared by intramolecular cross-linking. Therefore, peaks A and B are expected to be the peptides containing intramolecularly cross-linked sites. To identify the intramolecularly cross-linked sites, N-terminal amino acid sequences and molecular masses of these two peptides were determined (Table II). Two amino acid sequences were detected by Edman degradation of peptide peak A, which completely agreed with amino acid sequences from Tyr45-Lys49 and Ile237-Gln247 of the 33-kDa protein, in which only Lys48 and Glu246 were not detected. Peptide peak A had a measured mass of 1927.10 Da, which is consistent with the predicted mass of Tyr45-Lys49 plus Ile237-Gln247 minus one molecule of H2O (1927.18 Da). These results clearly indicate that peptide peak A is the intramolecularly cross-linked product between the amino group of Lys48 in Tyr45-Lys49 and the carboxyl group of Glu246 in Ile237-Gln247. The N-terminal sequence of peptide peak B agreed with Arg5-Lys20 of the 33-kDa protein, in which Glu10 and Lys14 were not detected. A measured mass of peptide peak B (1968.00 Da) was consistent with the predicted mass of Arg5-Lys20 minus one molecule of H2O (1968.20 Da). These indicate that peptide peak B is the intramolecularly cross-linked product between the carboxyl group of Glu10 and the amino group of Lys14 in Arg5-Lys20. These results indicate that amino acid residues between Lys48 and Glu246 and between Glu10 and Lys14 are closely associated with each other through electrostatic interaction.

Peptide peaks 2, 3, 4, and 6, which were significantly decreased by intramolecular cross-linking, were found to be Gly160-Lys186, Ile237-Gln247, Thr15-Lys44, and Leu77-Lys101, respectively, by mass spectrometric analysis (data not shown). Peptide peaks 3 and 4 are involved in the intramolecularly cross-linked peptide peaks A and B. The cross-linked products containing peptide peaks 2 and 6 were not, however, found on the peptide map of lysyl endopeptidase digests of the intramolecularly cross-linked 33-kDa protein (Fig. 5B). It is likely that these cross-linked products were retained in and not eluted from the reversed-phased HPLC column. Thus, in addition to the intramolecular cross-linking between Lys48 and Glu246 and between Glu10 and Lys14, intramolecular cross-linkings containing the peptide of Gly160-Lys186 and Leu77-Lys101 seem to be formed.

DISCUSSION

Reactivation Mechanism of Oxygen Evolution by Binding of the 33-kDa Protein—The present results demonstrated that the rebinding and manganese-stabilizing capabilities of the 33-kDa protein were retained but its reactivating ability was lost by intramolecular cross-linking of the protein with EDC. This implies that different mechanisms exist for the stabilization of manganese binding and reactivation of oxygen evolution; the latter but not the former was impaired by the intramolecular cross-linking. Several possibilities may be considered as responsible for loss of the reactivating ability, e.g. an inhibition of homodimerization of the 33-kDa protein which might be essential for its functioning, a loss of the function of the 33-kDa protein in maintaining binding of Ca2+ and/or Cl−, and a suppression of conformational changes of the 33-kDa protein possibly accompanying its binding to PSII. Our present results support the hypothesis that a conformational change occurred.
accompanying binding of the 33-kDa protein, and this conformational change was suppressed by the intramolecular cross-linking, since the secondary structure of the protein was significantly stabilized against heat treatment by intramolecular cross-linking (Fig. 4). This suggests that the structural flexibility of the 33-kDa protein was remarkably reduced. In addition, while urea/NaCl-washed PSII membranes reconstituted with the native 33-kDa protein completely rebound the 23-kDa protein, the PSII reconstituted with the intramolecularly cross-linked 33-kDa protein scarcely rebound the 23-kDa protein (data not shown). Since the 23-kDa protein cannot directly associate with the 33-kDa protein in solution (24), these results suggest that binding of the 33-kDa protein to PSII alters the conformation of the protein itself which is essential for binding of the 23-kDa protein. The occurrence of a structural change of the protein is consistent with results of pH-dependent structural changes (38), effects of genetic or chemical modification of its disulfide-forming cysteines (39, 40), or the effects of conformational constraints resulting from other amino acid substitutions (41).

From the present and previous results, however, we cannot determine whether the conformational change of the 33-kDa protein itself or a further structural rearrangement of intrinsic PSII proteins allosterically induced by binding of the 33-kDa protein is responsible for the reactivation of oxygen evolution. The possible structural changes of intrinsic PSII proteins upon binding of the 33-kDa protein have been previously reported; for example, we recently showed that removal of the 33-kDa protein makes the C-terminal region of CP43 accessible to trypsin, thus suggesting that removal of the protein at the luminal side induces a conformational change of the CP43 protein at the stromal side (42). An effect on properties of the acceptor side of PSII upon either biochemical removal of the 33-kDa protein (43) or genetic deletion of the psbO gene encoding the 33-kDa protein (44, 45) has also been reported based on thermoluminescence and fluorescence measurements. Based on these results, we propose the following mechanism for reactivation of oxygen evolution by binding of the 33-kDa protein: binding of the 33-kDa protein to PSII alters the conformation of the 33-kDa protein itself which allosterically results in structural changes of intrinsic PSII proteins ligating the manganese atoms, leading to formation of a functional conformation of the manganese cluster and then the reactivation of oxygen evolution.

**Structure of the 33-kDa Protein**—Although the primary structure of the 33-kDa protein has been determined in various species of plants (46), there is only very limited information concerning the tertiary structure of the protein. Two Cys residues (Cys10 and Cys51) of the protein form a disulfide bond important for maintaining the functional structure of the protein (40, 45, 47). The secondary structural analysis of the 33-kDa protein in solution by far-UV CD spectroscopy revealed that the protein contains a large proportion of β-sheet and a relatively small amount of α-helical structure (48). Recently, we reported that the positive charges of ε-amino groups on Lys4, Lys20, Lys66 or Lys76, Lys91, Lys105, Lys135, Lys159, Lys186 and one or two Lys in Lys230-Lys236 in the 33-kDa protein electrostatically interact with negative charges on PSII intrinsic proteins (27). This implies that these Lys residues are located on the surface of the protein which interacts with PSII intrinsic proteins when it binds to PSII. The present study revealed that the positive charges of the amino group of Lys48 and Lys142 electrostatically associate with the negative charges of the carboxyl group of GuH266 and Glu10, respectively, when the protein is free in solution. This is very interesting, since we have reported that the carboxyl group of Glu246 or C terminus electrostatically interacts with the amino group of Lys190 of the 33-kDa protein when the protein is associated with PSII (49). This indicates that the carboxyl group in the C-terminal region of the 33-kDa protein electrostatically interacts with different amino groups, depending on whether the protein is free in solution or binds to PSII, which again suggests that the 33-kDa protein alters its conformation upon binding. Fig. 6 summarizes the localization of amino acid residues interacting with each other in the spinach 33-kDa protein which have been determined in the present and previous studies (27, 40, 47). Amino acids associated with each other through electrostatic interaction or disulfide bond are connected with a solid line when the protein is free in solution and with a dotted line when the protein is associated with PSII. Domains involving Lys residues interacting with PSII intrinsic proteins are boxed. These results provide an important information for the tertiary structure of the 33-kDa protein.

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