Evidence from Crosslinking for a Close Association of the Extrinsic 33 kDa Protein with the 9.4 kDa Subunit of Cytochrome b 559 and the 4.8 kDa Product of the psb I Gene in Oxygen-Evolving Photosystem II Complexes from Spinach

Isao Enami¹, Sachiko Ohta¹, Satoru Mitsuhashi¹, Seitaro Takahashi¹, Masahiko Ikeuchi² and Sakae Katoh³

¹Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo, 162 Japan
²Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, 351-01 Japan
³Department of Botany, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113 Japan

Oxygen-evolving photosystem II complexes from spinach, which lack light-harvesting chlorophyll a/b proteins, were treated with a bifunctional crosslinking reagent, hexamethylene-diisocyanate. Identification of crosslinked proteins with antisera raised against various constituent proteins of the oxygen-evolving PS II complex showed that the extrinsic 33 kDa protein is located less than 11 Å from the 9.4 kDa subunit of cytochrome b 559 and the 4.8 kDa product of psb I gene.

Key words: Crosslinking — Cytochrome b 559 — Extrinsic 33 kDa protein — Oxygen evolution — psb I gene product.

The extrinsic 33 kDa protein, which is associated with PS II reaction center complexes on the luminal side of the thylakoid membrane, plays an important role in the water oxidation. The protein is needed to maintain the functional conformation of the Mn cluster (Ono and Inoue 1984a, b, 1985, Miyao and Murata 1984b, Kuwabara et al. 1985) and its solubilization from the complexes is accompanied by a concomitant inactivation of oxygen evolution (Ono and Inoue 1983, 1984b, Miyao and Murata 1984b, c). Various attempts have been made to identify the subunit protein(s) of the PS II core complex with which the 33 kDa protein is associated. Trypsin-digestion experiments have suggested that the 43 kDa chlorophyll-carrying protein (CP43) is shielded by the 33 kDa protein against proteolytic attack (Isogai et al. 1985). Binding of the 33 kDa protein to another chlorophyll-carrying protein of 47 kDa (CP47) has been demonstrated with various bifunctional crosslinkers (Enami et al. 1987, 1989a, 1990, Bricker et al. 1988). Recent experiments showed that all the 33 kDa molecules can be covalently linked to CP47 at a one-to-one ratio without any significant effect on the oxygen-evolving activity (Enami et al. 1991). The extrinsic 23 kDa protein was shown to be in contact with the 33 kDa protein (Miyao and Murata 1983, Andersson et al. 1984, Enami et al. 1990). Results of photoaffinity crosslinking led to the conclusion that the 33 kDa protein is associated with the D1 and D2 proteins and with a 34 kDa protein (Mei et al. 1989). The binding of the 33 kDa protein to the D1 and D2 proteins was also suggested by the results of affinity chromatography with the 33 kDa protein as a ligand (Isogai et al. 1987, Gounaris et al. 1988).

The PS II reaction center complex also contains several small subunits. In particular, the 9.4 kDa and 4.4 kDa subunits of cytochrome b 559 and the 4.8 kDa polypeptide, which was identified as the product of the psb I gene (Ikeuchi and Inoue 1988a), are tightly associated with the D1/D2 complex (Nanba and Sato 1987, Ikeuchi and Inoue 1988a, b). However, no crosslinked product

Abbreviations: LHC II, light-harvesting chlorophyll a/b protein of PS II; HMDI, hexamethylene-diisocyanate; CBB, Coomassie brilliant blue R-250; HTG, n-heptylthioglucoside; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; CP 29, a minor antenna chlorophyll a/b protein of PS II.
containing any of these small polypeptides has yet been reported. The spatial arrangement of these small intrinsic polypeptides in the PS II core complex remains to be determined.

In the present study, crosslinking of oxygen-evolving PS II complexes from spinach was carried out with hexamethylene-diisocyanate (HMDI), which interacts with a wide range of functional groups on amino acids. Evidence was obtained indicating that a nearest-neighbor relationship exists between the 33 kDa protein and either the 9.4 kDa subunit of cytochrome b 559 or the 4.8 kDa product of the psb I gene.

Materials and Methods

Oxygen-evolving PS II complexes, which were largely depleted of LHC II but still retained the three extrinsic proteins of 33, 23 and 17 kDa, were prepared from spinach with a non-ionic detergent, /i-heptylthioglucoside (HTG), as described previously (Enami et al. 1989b, 1990). The complexes were treated with 1.5 M NaCl under room light for 30 min to deplete the 23 and 17 kDa proteins (Miyao and Murata 1983). Washing with CaCl₂ was carried out as described previously (Ono and Inoue 1983).

For crosslinking, indicated amounts of HMDI were added to HTG-purified complexes (0.8 mg Chl ml⁻¹) which had been suspended in 40 mM MES-NaOH (pH 6.5), 10 mM NaCl, 5 mM MgCl₂ and 0.4 M sucrose. The crosslinker was freshly dissolved in dimethylsulfoxide prior to each experiment. After incubation for 10 min at room temperature, the crosslinking reaction was terminated by addition of 0.5 M glycine. Polypeptides of the crosslinked complexes were analysed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (1970). Western blotting was conducted as described previously (Enami et al. 1990) with antisera raised specifically against CP47, CP43, LHC II, the extrinsic 33 kDa protein (Aoki et al. 1986), the D1 and D2 proteins, the large subunit of cytochrome b 559 (9.4 kDa polypeptide) and the product of the psb I gene (4.8 kDa polypeptide) (Ikeuchi and Inoue 1988b), all from spinach.

Results

Figure 1 shows CBB-stained polypeptide bands resolved from HTG-purified complexes treated with different concentrations of HMDI. The positions of the bands of apoproteins of CP47, CP43 and CP29, the extrinsic 33 kDa protein, the D1 and D2 proteins and the 9.4 kDa subunit of cytochrome b 559 are indicated in lane 1 (Enami et al. 1989b). The 4.8 kDa product of the psb I gene (Ikeuchi and Inoue 1988a, b) gave only a diffuse and very weakly stained band under the electrophoretic conditions used. The position of the polypeptide band was more clearly visualized by immunoblotting with a specific antiserum (see Fig. 2). The extrinsic 23 and 17 kDa proteins were not seen because the complexes had been washed with 1.5 M NaCl prior to crosslinking (Miyao and Murata 1984a).

After treatment with HMDI, the intensity of most of the protein bands decreased and the upper region of the gel was entirely stained with CBB, an indication of the migration of various crosslinked products. The amount of protein that remained at the interface between the stacking and resolving gels increased. However, no distinct bands of products were resolved in the high molecular-mass regions. By contrast, two new bands, labelled A and B, appeared between the bands of CP43 and the 33 kDa protein. Crosslinking of unwashed complexes also yielded these two bands but the resolution was poorer than when NaCl-washed complexes were analyzed (data not shown), presumably because of comigration of a product(s) involving the 23 and/or 17 kDa proteins. Therefore, all the subsequent experiments were carried out with NaCl-washed complexes. Unwashed preparations did, however, yield essentially the same results with respect to the two bands of interest.
For identification of the crosslinked proteins, immunoblotting was carried out with eight antisera raised against the apoproteins of CP47, CP43 and LHCII, the extrinsic 33 kDa protein, the D2 and D1 proteins, the 9.4 kDa subunit of cytochrome $b_559$ and the 4.8 kDa product of the psb$I$ gene (Fig. 2). As shown in panel A, all the antisera used were monospecific, apart from the antiserum against LHC II (anti-LHC II) which crossreacted with CP29 (lane 6) and the antiserum against the 9.4 kDa subunit (anti-9.4) which generated a weak band at about 50 kDa (lane 7). The band of 63 kDa in lanes 4 and 5 was a heterodimer of the D1 and D2 proteins (Nanba and Satoh 1987).

Panel B of Figure 2 shows an immunoblot of HTG complexes that were treated with 0.08% HMDI. In contrast to the CBB-stained gel, no significant bands were generated in the upper region of the gel. After more extensive immunostaining reactions, very diffuse blots appeared in lanes treated with the antisera against CP47 (anti-47), CP43 (anti-43), the D1 protein (anti-D1) and the D2 protein (anti-D2) (data not shown). A diffuse band appeared above CP47 (lane 1) and CP43 (lane 2). Because no band was generated at the corresponding positions in other lanes, these bands are ascribed to modification or intramolecular crosslinking of CP47 or CP43 by HMDI.

Note that two sharp bands, which reacted with the antiserum against the 33 kDa protein (anti-33), appeared at the positions that corresponded to bands A and B (lane 3). Re-electrophoresis of bands A and B excised from CBB-stained gels and subsequent immunoblotting confirmed that the two bands that reacted with anti-33 were indeed bands A and B (data not shown). The apparent molecular masses of bands A and B were 41 and 38 kDa, respectively, suggesting that the two bands are crosslinked products of the 33 kDa protein and a small polypeptide of less than 10 kDa. In fact, a band that reacted with the antiserum against the 4.8 kDa polypeptide (anti-4.8) appeared at a position corresponding to band B in lane 8. We conclude, therefore, that the lower band of a 38 kDa product is due to crosslinking of the 33 kDa protein with the 4.8 kDa product of the psb$I$ gene (also see below).

The situation was more complicated in the case of band A because both anti-D1 (lane 5) and anti-9.4 (lane 7) yielded a band at corresponding positions on the gel. However, the apparent molecular mass of band A seems to be inconsistent with crosslinking of the 33 kDa protein and the

![Fig. 2 Immunoblot analysis of crosslinked products. A, NaCl-washed HTG complexes. B, NaCl-washed HTG complexes treated with 0.08% HMDI. Lane 1, reaction with anti-47; lane 2, with anti-43; lane 3, with anti-33; lane 4, with anti-D2; lane 5, with anti-D1; lane 6, with anti-LHC II; lane 7, with anti-9.4; lane 8, with anti-4.8. Arrowheads indicate bands of crosslinked products. Arrows show gel positions to which respective immunoreactive proteins would be expected to migrate.]
D1 protein, or of the two proteins plus the 9.4 kDa subunit. It is more likely that band A is a product of crosslinking either between the 33 kDa protein and the 9.4 kDa subunit or between the D1 protein and the 9.4 kDa subunit. To distinguish between these two possibilities, crosslinking experiments were carried out with HTG complexes, from which the 33 kDa protein had been extracted by washing with CaCl\textsubscript{2} (Ono and Inoue 1983) (Fig. 3). As expected, removal of the 33 kDa protein resulted in disappearance of all the bands reactive with anti-33, i.e., the band of the 33 kDa protein itself and the bands of its two crosslinked products (panel II, lane 1). The product that reacted with anti-9.4 also disappeared (lane 3). However, the product that reacted with anti-D1 remained unaffected (lane 2). In addition, in this particular experiment, the band that reacted with anti-D1 migrated slightly more rapidly than the bands that reacted with anti-33 or anti-9.4. We conclude, therefore, that band A is a composite of two products: the 33 kDa protein crosslinked with the 9.4 kDa subunit of cytochrome \textit{b} 559 and the D1 protein crosslinked with an unidentified polypeptide. Note that the removal of the 33 kDa protein also resulted in disappearance of the product that reacted with anti-4.8 (lane 4). This result supports our conclusion that band B contains the product of crosslinking between the 33 kDa protein and the 4.8 kDa product of the \textit{psb} \textit{I} gene.

**Discussion**

The present report describes two novel crosslinked products generated from constituent proteins of spinach oxygen-evolving PS II complexes; the first product is the 33 kDa protein crosslinked with the 9.4 kDa subunit of cytochrome \textit{b} 559 and the second is the 33 kDa protein covalently bound to the 4.8 kDa product of the \textit{psb} \textit{I} gene. These crosslinked products were not detected in the previous studies which, instead, showed that the 33 kDa protein preferentially crosslinks with CP47 (Enami et al. 1987, 1989a, 1990, 1991, Bricker et al. 1988). The difference can be ascribed to differences between the crosslinkers used. Crosslinking of the 33 kDa protein and CP47 was achieved with succinimidyl reagents which are specific for amino group of lysine and with EDC which reacts with carboxyl and amino groups. By contrast, HMDI reacts with a wider variety of functional groups, such as amino, sulfhydryl, imidazole, aromatic hydroxyl and carboxyl groups (Means and Feeeney 1971). Thus, the successful crosslinking of the 33 kDa protein with the small polypeptides can be ascribed to the broader specificity of the crosslinker employed in the present work.

The product formed by crosslinking between the 33 kDa protein and CP47 was not detected in the present study. Previous experiments showed that the chain length of the crosslinker is an important factor that affects the crosslinking of the two proteins. The best reagent is the zero-length crosslinker, EDC, which allows 100% crosslinking of the 33 kDa protein with CP47 (Enami et al. 1991). The yield of the crosslinked product decreased as the chain length of bifunctional succinimidyl reagents was increased from 6 Å to 11 Å and fell to zero at 14 Å (Enami et al. 1990). HMDI which has a chain length of 11 Å can, therefore, be expected to act in this situation as a poor crosslinker. Another aspect of our results to be considered is the observation that, although CBB-stained gels had very diffuse bands in the large molecular-mass region, none of the antisera used generated significant bands in this region. Only after prolonged development of color did diffuse and weak bands appear in lanes reacted with several antisera. This result suggests that the immunological reactivity of the crosslinked proteins is considerably diminished by treatment with HMDI, probably as a result of blockage of the functional groups of various amino acid residues. The absence of no distinct products in the upper parts of

---

**Fig. 3** Immunoblot analysis of crosslinked products from HTG complexes depleted of the 33 kDa protein. The concentration of HMDI was 0.08%. I, NaCl-washed HTG complexes; II, HTG complexes washed with 1.0 M CaCl\textsubscript{2}. Lane 1, reaction with anti-33; lane 2, with anti-D1; lane 3, with anti-9.4; lane 4, with anti-4.8. For explanations of arrowheads and arrows, see the legend to Fig. 2.
CBB-stained gels may also be ascribable to variations in the extent of modification of functional groups and intramolecular crosslinking of proteins by HMDI. The two crosslinked products obtained in the present work appear to be exceptional in this respect. At any event, crosslinking patterns vary greatly depending upon crosslinking reagents used. Thus, the choice of an appropriate reagent is important for crosslinking experiments designed to determine nearest-neighbor relationships for particular proteins.

The amino acid sequences of the 9.4 kDa subunit of cytochrome \( b \) 559 (Herrmann et al. 1984, Cramer et al. 1986) and the 4.8 kDa polypeptide (Ikeuchi and Inoue 1988a, b) indicate that both polypeptides have a hydrophobic region which is assumed to span the thylakoid membrane. The carboxyl-terminal sequence (residue 45 to the C-terminus) of the 9.4 kDa subunit, which is exposed to the lumenal space, contains 9 amino acid residues that could react with HMDI (Asp 45, Glu 54, Tyr 55, Glu 58, Asp 71, Glu 74, Asp 77, Glu 78 and the carboxyl-terminus; Herrmann et al. 1984, Widger et al. 1985, Vallon et al. 1989). The amino- and carboxyl-terminal sequences of the 4.8 kDa polypeptide, which also extend beyond the membrane, have four amino acid residues that could react with HMDI: Lys 5 in the amino-terminal region and Asp 27, Glu 35 and Glu 36 in the carboxyl-terminal region (Ikeuchi and Inoue 1988a, b). Thus, HMDI can crosslink the polypeptides to the 33 kDa protein by reacting with any one of these functional groups. Because HMDI has a maximum chain length of 11 Å, the distance between the 33 kDa protein and either of the two small polypeptides must be less than 11 Å. The functions of cytochrome \( b \) 559 and the product of the \( psb I \) gene are not known. The results obtained here suggest that the two polypeptides may have a structural role in binding the 33 kDa protein to the PS II complex.

As stated in the Introduction, there are indications that the 33 kDa protein is also closely associated with CP47, CP43 and the D1 and D2 proteins. Thus, all the major constituent subunits of the PS II reaction center complex are located near or in contact with the 33 kDa protein. This geometry reflects a situation wherein all the intrinsic proteins of the PS II core complex span the thylakoid membrane more or less in parallel and the 33 kDa protein covers the surface of the complex exposed to the lumenal space (Haag et al. 1990). Such a molecular arrangement would be important for the stability and function of the 33 kDa protein in the PS II complex. Figure 4 shows a schematic representation of the possible molecular organization of the oxygen-evolving PS II reaction center complex, constructed by taking into account the results of the crosslinking experiments carried out to date. At present, the arrangement of the intrinsic proteins with respect to one another is unknown for the most part. It remains to be determined whether the 33 kDa protein is also associated with the 4.4 kDa subunit of cytochrome \( b \) 559 or with other small subunits that were recently reported to be present in oxygen-evolving PS II complexes (Ikeuchi and Inoue 1988b, Ikeuchi et al. 1989a, b). Further experiments with various crosslinkers can be expected to provide important information as to the molecular organization of the PS II complex.

We thank Prof. T. Horio and Dr. T. Kakuno, Osaka University, for generously providing us with antisera against CP47, CP43, LHC II and the extrinsic 33 kDa protein.

Reference


Aoki, K., Ideguchi, T., Kakuno, T., Yamashita, J. and Horio, T.


(Received October 14, 1991; Accepted February 6, 1992)