Biochimica et Biophysica Acta, 973 (1989) 35–40
Elsevier

BBA 42905

Nearest neighbor relationships among constituent proteins of oxygen-evolving Photosystem II membranes: binding and function of the extrinsic 33 kDa protein

Isao Enami [1], Takeshi Miyaoka [1], Yasuki Mochizuki [1], Jian-Ren Shen [2], Kazuhiko Satoh [2] and Sakae Katoh [2]

[1] *Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, and* [2] *Department of Botany, Faculty of Science, University of Tokyo, Hongo, Tokyo (Japan)*

(Received 29 August 1988)

Key words: Crosslinking; Nearest neighbor relationship; Extrinsic 33 kDa protein; Oxygen evolution; Mn cluster

Nearest neighbor relationships among constituent proteins of oxygen-evolving Photosystem II (PS II) membrane preparations from spinach were investigated by means of crosslinking with a cleavable bifunctional crosslinker, dithiobis(succinimidylpropionate) (DSP). (1) Diagonal gel electrophoresis revealed crosslinking between two extrinsic proteins of 17 and 23 kDa, between the extrinsic 33 kDa protein and the 47 kDa chlorophyll-carrying protein and between the 26 and 27 kDa apoproteins of light-harvesting chlorophyll *a*/*b* protein. In addition, a product which involved a protein of 29 kDa was detected. (2) Amounts of the extrinsic proteins crosslinked were determined by washing DSP-treated membranes with high concentrations of urea and NaCl, or $CaCl_2$. Neither of the two extrinsic proteins of 17 and 23 kDa was crosslinked with intrinsic membrane proteins, whereas 15 to 20% of the 33 kDa protein was immobilized by treatment with 0.1% DSP. Oxygen evolution became resistant to the urea/NaCl-wash proportionally to the amount of the 33 kDa protein crosslinked, indicating that the 33 kDa protein covalently bound to the 47 kDa protein is still fully functional. (3) The crosslinking of the 33 kDa protein accompanied by parallel increases in the amount of Mn remained unextracted, and the rate of oxygen evolution survived after 30-min treatment of PS II membranes at pH 9.0. Thus the 33 kDa protein has a protective effect on the Mn cluster at the alkaline pH. In contrast, Mn was mostly extracted with a high concentration of Tris, irrespective of the crosslinking of the 33 kDa protein.

Introduction

Three extrinsic proteins of 17, 23 and 33 kDa associated with the inner surface of the thylakoid membranes play important roles in photosynthetic oxygen evolution of higher plants (for reviews, see Refs. 1–4). The 17 and 23 kDa proteins are considered to have regulatory roles in oxygen evolution by providing appropriate ionic environments for the catalytic Mn cluster. The two proteins, which are selectively solubilized with high concentrations of NaCl [5–8], are functionally replaced at least partly by Ca^{2+} and Cl^- [9,10]. The 33 kDa protein is more indispensable: there is a consensus that the protein is needed to maintain the functional conformation of the Mn cluster [11–15]. The 33 kDa protein, together with the 17 and 23 kDa proteins, is solubilized by treatments of PS II membranes with a high concentration of Tris [16] or at alkaline pH [17] concomitant with release of Mn, while treatment with 1 M $CaCl_2$ [18] or with 2.6 M urea in the presence of 0.2 M NaCl [13] releases the three proteins leaving the Mn cluster unextracted.

The association of the 17, 23 and 33 kDa proteins with oxygen-evolving membranes has been investigated by reconstitution experiments [7,11,14,19], proteinase digestion [20,21], immunoprecipitation [22,23] and crosslinking [24–31]. However, an important question as to which membrane proteins the three extrinsic proteins attach to remains to be studied. A promising approach to the question is crosslinking with a cleavable bifunctional reagent, because crosslinked proteins are readily identified by cleaving the crosslinker. Using

Abbreviations: DSP, dithiobis(succinimidylpropionate); PS, Photosystem; LHCP, light-harvesting chlorophyll *a*/*b* protein of PS II; Mes, 4-morpholineethanesulfonic acid.

Correspondence: I. Enami, Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo 162, Japan.

highly purified oxygen-evolving PS II complexes, we have recently shown that the 33 kDa protein crosslinks with the 47 kDa chlorophyll-carrying protein with DSP, a bifunctional reagent with a disulfide bond in the middle of the molecule [30]. The crosslinking between the two proteins was later confirmed by Bricker et al. [31].

In the present work, crosslinking experiments were extended to oxygen-evolving PS II membranes to determine the nearest neighbor relationship among proteins involved in oxygen evolution. The crosslinked membranes were subjected to various treatments, which liberate the extrinsic proteins, to examine the binding and function of the 33 kDa protein. A part of the present work has appeared in Ref. 29.

Materials and Methods

Oxygen-evolving PS II membranes were prepared from spinach chloroplasts as in Ref. 17, except that a Triton X-100/chlorophyll ratio of 15 : 1 was used. The preparations were suspended in 40 mM Mes-NaOH (pH 6.5), 0.4 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl.

For crosslinking, indicated amounts of a freshly prepared dimethyl sulfoxide solution of DSP (10 mg/ml) were added to sample suspensions containing 1 mg chlorophyll per ml. After incubation for 10 min at room temperature, the crosslinking reaction was terminated by adding glycine in 100-times molar excess over the crosslinker. Proteins crosslinked were analyzed by two-dimensional electrophoresis as described in Ref. 30. Acrylamide concentrations were 9.5–11.5% and 11.5% for the first- and second-dimensional electrophoresis, respectively. Both gels contained 6 M urea.

For determination of the extrinsic proteins crosslinked DSP-treated membranes were incubated with 2.6 M urea plus 0.2 M NaCl [13], 1 M $CaCl_2$ [18], 0.8 M Tris-HCl (pH 8.0) [16] or 0.1 M glycine-NaOH (pH 9.0) [17] for 30 min at 0 °C. After centrifugation at 35 000 × *g* for 10 min, the membranes pelleted were once washed and then treated with 8 M urea, 10% SDS and 10% 2-mercaptoethanol for 30 min at 25 °C. Polypeptide compositions of the membranes were analyzed by electrophoresis on polyacrylamide gels containing 6 M urea according to the method of Laemmli [32]. Acrylamide concentrations were 4.5% for the stacking and 11.5% for the resolving gels. Gels were stained with Coomassie brilliant blue R-250 and scanned at 555 nm with a Shimadzu CS-910 chromatoscanner. Amounts of the 33 kDa protein crosslinked were estimated by measuring peak areas of the protein relative to that of the 47 kDa protein.

Oxygen evolution was measured with a Clark-type oxygen electrode at 27 °C as in Ref. 33. The reaction medium contained 0.4 M sucrose, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM NaCl, 2 mM 2,5-dimethyl-*p*-benzoquinone, 40 mM Mes-NaOH (pH 6.5) and PS II membranes (10 μg chlorophyll per ml). Rates of oxygen evolution in PS II membranes used here ranged from 500 to 600 μmol O_2 per mg chlorophyll per h. The abundance of Mn was determined with a Shimadzu atomic absorption spectrophotometer (AA640-01) equipped a graphite furnace atomizer (GFE-2). Chlorophyll concentration was determined according to the method of Arnon [34].

Results

Identification of crosslinked products

Polypeptide patterns of oxygen-evolving PS II membranes treated with different concentrations of DSP are presented in Fig. 1. Lane 1 shows three extrinsic proteins of 17, 23 and 33 kDa extracted with 0.8 M Tris-HCl (pH 8.3) [16]. The three proteins were clearly resolved, together with the 43 and 47 kDa chlorophyll-carrying proteins and large amounts of LHCP apoproteins of 26 and 27 kDa, from untreated PS II membranes (lane 2). Three new bands, which are labelled A, B and C, appeared on treatment of PS II membranes with DSP. The three bands were intensified as the crosslinker concentration was raised. Band B consisted of two closely migrating bands. Crosslinking of NaCl-washed membranes produced bands B and C but no band A (Fig. 1-II). This suggests that band A is related to the 17 and 23 kDa proteins which are removed by the NaCl-wash [6].

For identification of the crosslinked proteins, DSP-treated membranes were subjected to diagonal gel electrophoresis (Fig. 2). DSP has a disulfide bond in the middle of the symmetrical molecule. After the first one-dimensional electrophoresis, the gel was treated with 2-mercaptoethanol to cleave the disulfide bond of the crosslinker and then electrophoresed in the second dimension. Non-crosslinked proteins migrated diagonally, while crosslinked proteins gave rise to off-diagonal spots. Untreated PS II membranes yielded few off-diagonal spot (not shown). As shown in Fig. 2, however, a number of spots appeared from DSP-treated membranes. Band A yielded the 17 and 23 kDa proteins after cleavage of the crosslinker. This is consistent with the above observation that no band A appeared on treatment of NaCl-washed preparations with DSP (Fig. 1-II). An apparent molecular mass of band A was 44 kDa, which roughly corresponds to the sum of molecular mass of the 17 and 23 kDa proteins. A faint spot appeared between the 17 and 23 kDa proteins is ascribed to a protein comigrating with band A because it was seen in the two dimensional electrophoresis of the NaCl-washed preparations (Fig. 2-II). We conclude therefore that band A is a crosslinked product of the extrinsic 17 and 23 kDa proteins.

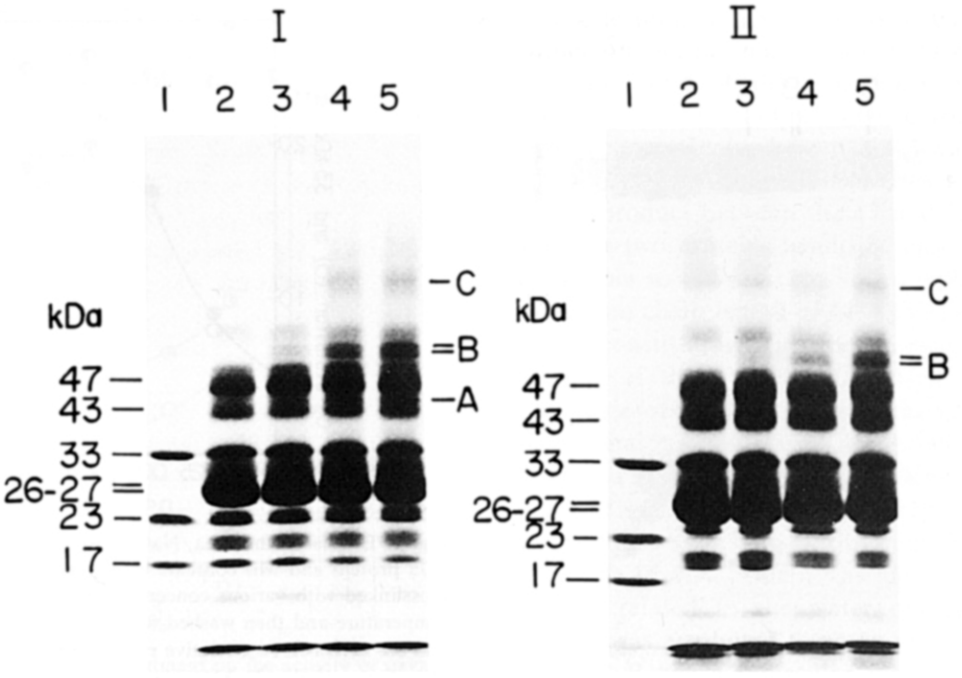

Fig. 1. Polypeptide patterns of DSP-treated oxygen-evolving PS II membranes. (I) Oxygen-evolving PS II membranes. (II) PS II membranes washed with 1 M NaCl. Lane 1, three extrinsic proteins extracted with 0.8 M Tris (pH 8.3) from oxygen-evolving PS II membranes; lane 2, non-crosslinked PS II membranes; lanes 3, 4 and 5, PS II membranes, to which 0.004% DSP was added 2, 4 and 6 times, respectively, at 10 min intervals.

The two bands, which are collectively called band B, migrated in the molecular mass region of 55–57 kDa. The most conspicuous off-diagonal spot appeared at the position corresponding to band B. The spot, however, consisted of at least three proteins, a protein of about 29 kDa which originated from band B, and two proteins of 26 and 27 kDa which were derived from a more diffuse product band. Note that there were various sizes of crosslinked products, all of which yielded the 26 and 27 kDa proteins after cleavage of the crosslinker. The two proteins are both ascribed to apoproteins of LHCP. Comigration of a crosslinked product of the LHCP apoproteins strongly interfered with identification of a partner crosslinked with the 29 kDa protein. Composition of band B was not further studied.

Although several off-diagonal spots appeared in the band C region, the pattern of the 33 kDa spot with a faint tailing matched only that of the 47 kDa spot. We attribute band C to a crosslinked product between the extrinsic 33 kDa protein and the chlorophyll-carrying 47 kDa protein. Crosslinking of the proteins has previously been observed in DSP-treated oxygen-evolving PS II complexes and the apparent molecular mass of the crosslinked product (79–83 kDa) [30] agrees with that of band C. The 47 kDa spot was considerably larger than the 33 kDa spot, but this can be ascribed to comigration of a 47 kDa protein dimer with band C [30].

The amount and function of the 33 kDa protein crosslinked

Amounts of the 33 kDa protein crosslinked were determined by washing DSP-treated PS II membranes with 2.6 M urea in the presence of 0.2 M NaCl. This treatment solubilizes the three extrinsic proteins without extracting Mn [13]. Fig. 3 shows that about 95% of the 33 kDa protein were extracted from untreated membranes by the urea/NaCl-wash. When PS II membranes were treated with increasing concentrations of DSP, the amount of the 33 kDa protein that remained unextracted progressively increased, and about 15% of the protein was immobilized with 0.075–0.1% DSP. The 33 kDa protein crosslinked at higher concentrations of DSP could not be determined accurately because significant amounts of polypeptides remained on the top of gels even after treatment with 2-mercaptoethanol. Presumably, polypeptides became strongly hydrophobic and aggregated when amino groups had been blocked.

Oxygen evolution was little affected by treatment of PS II membranes with 0.075% DSP (Table I). This, together with the following observation, indicates that the 33 kDa protein crosslinked with the 47 kDa protein fully retains its functional conformation. Oxygen evolution became partly resistant to the urea/NaCl-wash after DSP treatment of PS II membranes (Fig. 3). Provided that the activity was determined in the presence of sufficient concentrations of Ca^{2+} and Cl^-, the

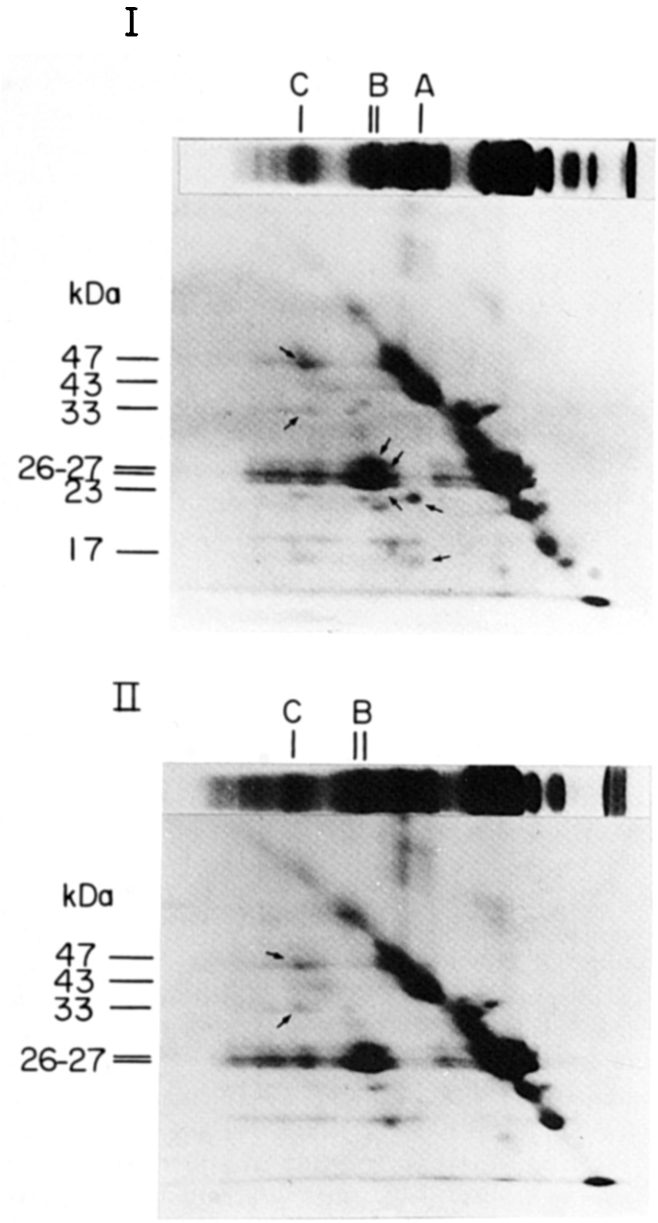

Fig. 2. Diagonal gel electrophoresis of oxygen-evolving PS II membranes. (I) Oxygen-evolving PS II membranes, to which 0.004% DSP was added six times at 10 min intervals. (II) NaCl-washed PS II membranes treated with DSP as in I. Polypeptide patterns of the one-dimensional gels were shown on the top of figures. The direction of migration is from left to right for the first one-dimensional, and from the top downward for the second-dimensional electrophoresis. Arrows indicate spots discussed in the text.

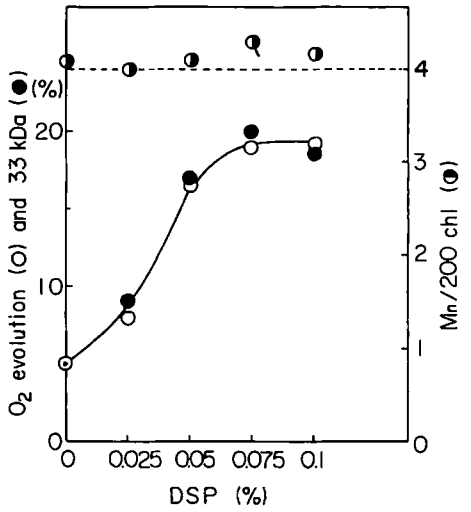

Fig. 3. Effects of the urea/NaCl-wash on oxygen evolution, the 33 kDa protein and Mn contents of oxygen-evolving PS II membranes crosslinked with various concentrations of DSP for 10 min at room temperature and then washed with a medium containing 2.6 M urea and 0.2 M NaCl. (○), relative rates of oxygen evolution; (●), relative abundance of the 33 kDa protein; (◑), Mn contents. The rate of oxygen evolution (520 μmol O_2 per mg chlorophyll per h) and the abundance of the 33 kDa protein in the original PS II membranes were taken as 100%.

rate of oxygen evolution survived the urea/NaCl-wash was well related to the amount of the 33 kDa protein crosslinked. On the other hand, the urea/NaCl-treatment totally washed out the 17 and 23 kDa proteins from DSP-crosslinked membranes, indicating that neither of the two proteins was covalently bound to membrane proteins with the crosslinker.

Similar results were obtained by washing DSP-treated membranes with 1 M $CaCl_2$ which solubilizes the extrinsic proteins but not Mn [18]. The crosslinking of the 33 kDa protein appreciably increased the rate of oxygen evolution resistant to the $CaCl_2$ treatment (Table I).

In contrast to the urea/NaCl- or $CaCl_2$-wash, treatment of the PS II membranes at an alkaline pH releases not only the 33 kDa protein but also Mn [6,17]. Fig. 4 shows that incubation for 30 min of non-crosslinked membranes at pH 9.0 diminished both the oxygen-evolving activity and the Mn abundance by more than 90%. The treatment was, however, less effective in solubilizing the 33 kDa protein than the urea/NaCl- or $CaCl_2$-wash [6]. It is to be noted that crosslinking of the

TABLE I

Effects of Ca^{2+}, Tris or NH_2OH wash on oxygen evolution and contents of the 33 kDa protein and Mn in DSP-treated PS II membranes

The membranes were treated with 0.075% DSP for 10 min at room temperature and then washed with 1 M $CaCl_2$, 0.8 M Tris (pH 8) or 1.5 mM NH_2OH. The NH_2OH treatment was carried out for 30 min at 0 °C.

Treatments	Cross-linking	Oxygen evolution (μmol O_2/mg chlorophyll per h) (%)	33 kDa protein (%)	Mn/200 chlorophyll
Non-treatment	−	543 (100)	100	4.1
	+	540 (99)	100	4.3
$CaCl_2$	−	27 (5)	8	4.2
	+	71 (13)	30	4.1
Tris	−	0 (0)	3	0.38
	+	0 (0)	22	0.42
NH_2OH	−	5 (1)	52	0.80
	+	6 (1)	60	0.93

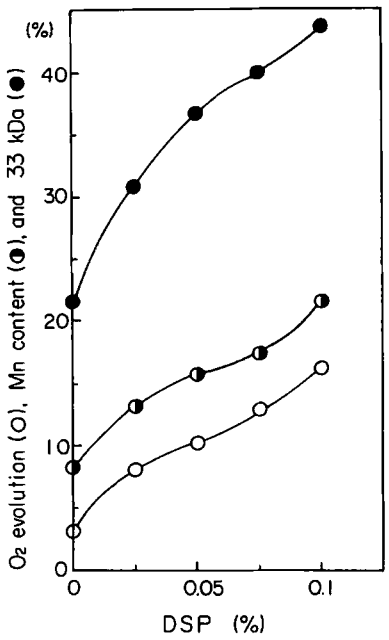

Fig. 4. Effects of an alkaline pH treatment on the activity of oxygen evolution and abundances of the 33 kDa protein and Mn in the PS II membranes treated with different concentrations of DSP. The oxygen-evolving PS II membranes were treated with indicated concentrations of DSP for 10 min and then incubated with 0.1 M glycine-NaOH (pH 9.0) for 30 min at 0 °C. Relative rates of oxygen evolution (○); relative abundances of the 33 kDa protein (●) and Mn (◑). The oxygen-evolving rates (520 μmol O_2 per mg chlorophyll per h) and the abundances of the 33 kDa protein and Mn in the PS II membranes before the alkaline pH treatment were taken as 100%.

33 kDa protein was accompanied by a parallel increase in the amount of Mn that remained unextracted by the alkaline pH-treatment. This shows that the 33 kDa protein has a protecting effect on the Mn cluster at an alkaline pH. There was also a good correlation between the rates of oxygen evolution that survived the alkaline pH-treatment and the amounts of unextracted Mn. This strongly suggests that all the remaining Mn atoms are organized into the functional cluster.

Treatment of PS II membranes with a high concentration of Tris at an alkaline pH is also known to liberate the 33 kDa protein and Mn [16]. The effect of the Tris-wash on DSP-treated membranes was, however, different from that of the alkaline pH-incubation. The crosslinked 33 kDa protein had no protecting effect on Mn in the presence of 0.8 M Tris (Table I). Thus the Tris-wash resulted in a total inactivation of oxygen evolution, irrespective of the binding of the 33 kDa protein. In this regard, the Tris-wash resembles the NH_2OH-wash, which extracted Mn, leaving a large fraction of the 33 kDa protein bound to the PS II membranes (Table I) [6].

Discussion

In the present work, four crosslinked products were obtained by treating PS II membranes with DSP, a cleavable bifunctional crosslinker. Crosslinked product A had a molecular mass of about 44 kDa and, when the disulfide bond of the crosslinker had been cleaved, produced the 17 and 23 kDa extrinsic proteins. No band A was obtained from NaCl-washed preparations which lack the two proteins. Thus band A is a crosslinked product between the 17 and 23 kDa protein. In situ, the two proteins would be closely associated with each other so as to be crosslinked with DSP which has a maximum chain length of 12 Å. The result is consistent with reconstitution experiments, which showed that the binding of the 23 kDa protein to the membranes is necessary for the association of the 17 kDa protein with its original binding site [7]. A crosslinked product containing a 16 and a 23 kDa protein from DSP-treated pea PS II preparations has been reported [26]. However, the product seems to be different from ours because, while the 16 kDa protein was identified as the extrinsic protein related to the water oxidation, the 23 kDa protein was attributed to an apoprotein of LHCP.

Band B has a molecular mass of 55–57 kDa and involves a protein of about 29 kDa. It is not clear why the product is split into closely migrating two bands. A candidate for the 29 kDa protein would be the apoprotein of CP29 [35]. Band B may be a crosslinked product between two molecules of the CP29 apoprotein or, more likely, the CP29 apoprotein crosslinked with an apoprotein of LHCP.

The 26 and 27 kDa apoproteins of LHCP are the most abundant proteins present in the membrane preparations (Fig. 1). Various sizes of crosslinked products between the two LHCP apoproteins were obtained, suggesting that DSP crosslinks LHCP in different oligomeric forms. The most predominant product, which comigrated with band B, may be a one to one crosslinked product of the two proteins. The crosslinking of LHCP apoproteins with dimethyladipimidate has been reported previously [24].

The fourth product is the 33 kDa protein bound to the chlorophyll-carrying 47 kDa protein. The crosslinking of the two proteins has been previously shown by diagonal electrophoresis of DSP-treated oxygen-evolving complexes [30]. The present work indicates that crosslinking of the two proteins is not an artifact introduced by the use of highly purified complexes which lack the 17 and 23 kDa proteins and LHCP. Of particular interest is the observation that oxygen evolution became resistant to the urea/NaCl-wash proportionally to the amount of the 33 kDa protein crosslinked. This indicates that the protein crosslinked is still fully functional. Thus, crosslinking of the 33 kDa protein provides a new approach for the binding and function of the protein. Earlier works have shown that two Mn atoms are gradually released from PS II membranes, from which the 33 kDa protein had been removed [12,13]. This indicates that, out of the four Mn atoms

present per PS II, two atoms bind more loosely to proteins than the other two and the 33 kDa protein has a protecting effect on the two loosely bound Mn atoms. An alkaline pH-treatment or Tris-wash solubilizes Mn, together with the 33 kDa protein [16,17]. The immobilization of the 33 kDa protein enabled us to examine whether release of Mn by these treatments is a consequence of the removal of the 33 kDa protein or not. The results show that the 33 kDa protein stabilizes the entire cluster consisting of four Mn atoms at pH 9.0. In contrast, irrespective of the crosslinking of the 33kDa protein, Mn was largely extracted by the Tris-wash, indicating that the solubilization of Mn with a high concentration of Tris is not a consequence of the removal of the 33 kDa protein.

The nearest neighbor relationship between the 33 kDa and the 47 kDa proteins implies that the 47 kDa protein might have an important function other than light-harvesting. It does not necessarily exclude, however, a possibility that the 33 kDa protein attaches to a membrane protein(s) other than the 47 kDa protein. The recent evidence shows that the D1 and D2 proteins constitute the functional core of PS II [36]. It is, therefore, interesting to examine whether or not the 33 kDa protein is associated with the D1 and/or D2 proteins. Experiments along this line are in progress.

Acknowledgement

This work was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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