Volume 226, number 1, 161–165 FEB 05421 December 1987

Crosslinking between the 33 kDa extrinsic protein and the 47 kDa chlorophyll-carrying protein of the PS II reaction center core complex

Isao Enami, Kazuhiko Satoh* and Sakae Katoh*

Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo 162 and **Department of Pure and Applied Sciences, College of Arts and Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan*

Received 4 September 1987; revised version received 26 October 1987

Using highly purified oxygen-evolving photosystem II complexes from spinach, the 33 kDa extrinsic protein was found to crosslink with the 47 kDa chlorophyll-carrying protein with a cleavable bifunctional crosslinking reagent, dithiobis(succinimidylpropionate).

Crosslinking; Reaction center complex; 33 kDa protein; 47 kDa protein; Oxygen evolution

1. INTRODUCTION

Photosynthetic oxygen-evolving complexes of chloroplasts involve three extrinsic proteins with molecular masses of about 33, 23 and 17 kDa, which are associated with the inner surface of thylakoid membranes [1–4]. The 33 kDa protein plays an important role in water oxidation [5,6]. Isolation of highly purified oxygen-evolving complexes from higher plants and a cyanobacterium [7–9] demonstrated that the 33 kDa protein is directly associated with the PS II reaction center complex, which consists of the 47 and 43 kDa chlorophyll-carrying proteins, the D-1 and D-2 proteins of about 30 kDa and the 9 kDa (and 5 kDa) apoprotein of cytochrome *b*-559 [10].

We have shown [11] that crosslinking of spinach oxygen-evolving membrane preparations with a bifunctional crosslinker yielded three major products, of which two were identified to arise from crosslinking between the 17 and 23 kDa extrinsic proteins and the 26 and 27 kDa apoproteins of LHCP. The third product contained the 33 kDa extrinsic protein and the 47 kDa subunit of the PS II reaction center complex. However, identification of the crosslinked proteins was somewhat obscured by comigration of, among others, large amounts of the crosslinked LHCP apoproteins.

Here we report crosslinking of the 33 kDa extrinsic protein with the 47 kDa chlorophyll-carrying protein. Interference from other crosslinked products could be minimized by the use of a highly purified oxygen-evolving complex with a simple subunit composition lacking LHCP and the 17 and 23 kDa extrinsic proteins [9]. Crosslinking of the complexes with a cleavable bifunctional reagent and two dimensional elec-

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

Abbreviations: PS II, photosystem II; LHCP, light-harvesting chlorophyll protein; DSP, dithiobis(succinimidylpropionate); Mes, 2-(*N*-morpholino)ethanesulfonic acid; CBB, Coomassie brilliant blue R-250

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

trophoresis of the crosslinked complexes after cleavage of the crosslinker in two different gel systems allowed us to identify the crosslinked proteins.

2. MATERIALS AND METHODS

Highly purified oxygen-evolving complexes, which are in essence the PS II reaction center complex with an attached 33 kDa protein, were prepared as in [9] with slight modifications. The spinach PS II membranes were solubilized with 60 mM β-octylglucoside, layered onto a discontinuous sucrose density gradient (10, 15, 20, 25 and 30% sucrose in a solution containing 60 mM β-octylglucoside, 10 mM NaCl and 20 mM Mes-NaOH, pH 6.5), then centrifuged at 313900 × *g* for 2 h. The complexes pelleted at the bottom of tubes were once washed with 40 mM Mes-NaOH (pH 6.5) containing 0.4 M sucrose and 10 mM NaCl, suspended in the same medium and stored at 77 K.

For crosslinking, the oxygen-evolving complexes containing 10–50 μg chlorophyll were well dispersed in 100 μl of 40 mM Mes-NaOH (pH 6.5), 10 mM NaCl, 5 mM $CaCl_2$ and 0.1% digitonin. After addition of 2 μl of a freshly prepared dimethyl sulfoxide solution of DSP (10 mg/ml), the suspension was incubated at room temperature for 10 min. The crosslinking reaction was terminated by addition of 100-times molar excess of glycine over the crosslinker. Then, 5 mM *N*-ethylmaleimide was added to block SH-groups of proteins.

Crosslinked proteins were analyzed by two-dimensional gel electrophoresis. The crosslinked complexes were treated with 8 M urea and 10% SDS for 30 min at room temperature. The first one-dimensional SDS gel electrophoresis was carried out according to Laemmli [12]. Acrylamide concentrations were 4.5% for the stacking gel and 11.5% or 9.5–11.5% for the resolving gel. Electrophoresis was carried out at a constant current of 8 mA at room temperature for 17 h. To cleave the disulfide bond of DSP, a 10 mm wide strip of the one-dimensional gel was incubated with a medium containing 125 mM Tris-HCl (pH 8.8), 10% 2-mercaptoethanol, 8 M urea and 10% SDS for 90 min at room temperature. The medium was renewed after each 30 min and pH of the last incubation medium was adjusted to 6.8. The strip was placed horizontally onto a slab gel (13 × 13 × 0.2 cm) containing 11.5% acrylamide and embedded with 1% agarose containing 125 mM Tris-HCl (pH 6.8) and 2% SDS. Electrophoresis in the second dimension was carried out at 8 mA for 17 h at room temperature. Gels were stained with CBB for proteins.

3. RESULTS

Fig.1 shows protein profiles of the purified oxygen-evolving complexes resolved by one-dimensional electrophoresis in 9.5–11.5% acrylamide gel containing 6 M urea. The complex consists of two chlorophyll-carrying proteins of 47 and 43 kDa, an extrinsic 33 kDa protein, the D-1 and D-2 proteins of 28–32 kDa and a 9 kDa apoprotein of cytochrome *b*-559 [9]. Due to large amounts of the samples loaded on gels, the D-1 and D-2 proteins were not well resolved (lane 1). There were several faint bands in the high-molecular-mass region.

Crosslinking of the purified complexes with 0.02% DSP resulted in significant diminution of the 47, 43 and 33 kDa bands and appearance of several crosslinked products in the upper part of the gels (lane 2). In particular, two closely migrating bands, labelled a and b, appeared in the 79–83 kDa region. Hereafter, we focus our attention on these two crosslinked products.

DSP is a bifunctional crosslinking reagent which reacts preferentially with amino groups [13]. Because the crosslinker has a disulfide bond in the middle of the symmetrical molecule, the crosslinked proteins could be identified by electrophoresis in the second dimension after cleavage of the disulfide bond. DSP has been used for crosslinking of pea and *Chlamydomonas* thylakoids [14,15].

All subunit proteins of non-crosslinked complexes migrated along a diagonal path (fig.2I). An exception is a spot, which appeared from a faint band in the upper region of the one-dimensional gel and had a molecular mass of 47 kDa. This indicates that a part of the 47 kDa protein migrates as a dimer in the first gel electrophoresis. The complexes, which had been crosslinked with DSP but not treated with 2-mercaptoethanol, gave a similar electrophoretic pattern (not shown). When the crosslinked complexes were run after cleavage of

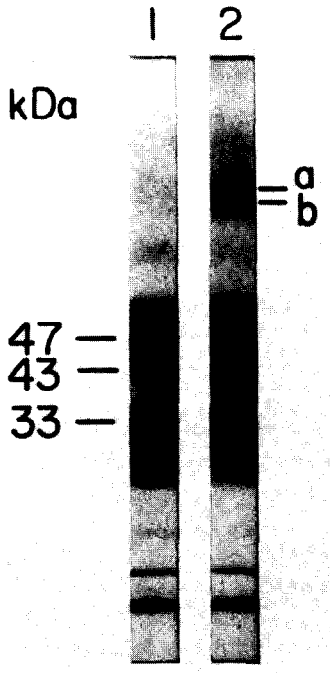

Fig.1. Effects of crosslinking on protein patterns of the oxygen-evolving complexes resolved by SDS-polyacrylamide gel electrophoresis. Gels contained 9.5–11.5% acrylamide and 6 M urea. Lane 1, untreated complexes; lane 2, complexes treated with 0.02% DSP.

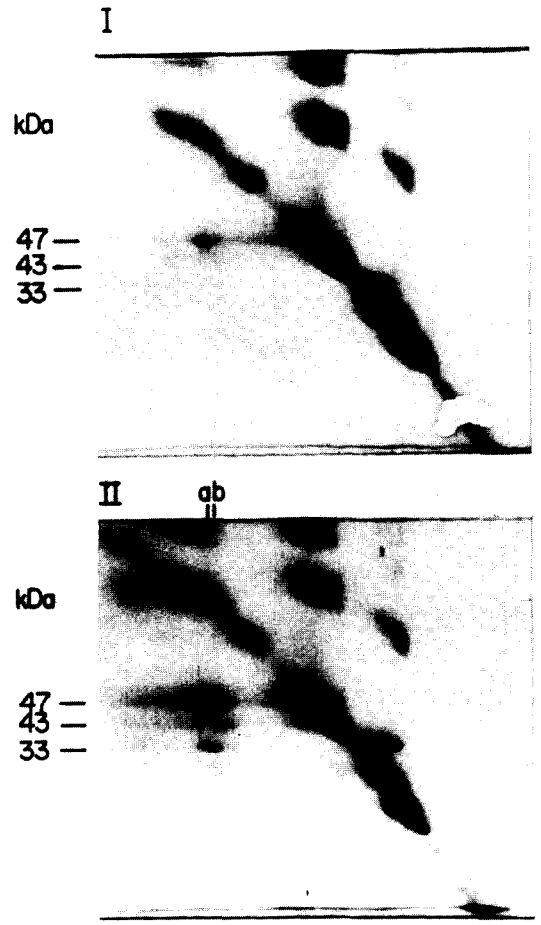

Fig.2. Diagonal electrophoresis of the oxygen-evolving complexes in gels containing 6 M urea. Acrylamide concentrations were 9.5–11.5% and 11.5% for the first- and second-dimensional electrophoresis. I, untreated complexes; II, complexes treated with 0.02% DSP. Proteins migrated from left to right in the first-dimensional, and from the top to bottom in the second-dimensional electrophoresis.

the disulfide bond of DSP with 2-mercapto-ethanol, several off-diagonal spots appeared (fig.2II). Both bands a and b produced two conspicuous spots having molecular masses of 47 and 33 kDa. Importantly, no other protein spots appeared at the positions corresponding to the two bands. An off-diagonal spot of 43 kDa and faint spots in the low-molecular-mass regions originated from crosslinked products which had migrated slightly more rapidly than the bands a and b in the first one-dimensional electrophoresis. The 43 kDa spot seems to have been derived from its crosslinked dimer, or a product containing the 43 kDa protein and a small protein. The results suggest that the bands a and b contain no other proteins but the 47 and 33 kDa proteins. However, the 47 kDa spot may as well be produced from a dimer of the 47 kDa protein which comigrates with the bands a and b in the first one-dimensional electrophoresis (fig.1 and fig.2I). Thus, the question remains as to whether or not the 47 kDa off-diagonal spot is derived from the crosslinked products.

We have searched for electrophoretic conditions which yield no dimer band of the 47 kDa protein and found that omission of urea from gels very much diminishes the dimer band. Fig.3I shows that non-crosslinked complexes produced only a barely detectable off-diagonal spot of the 47 kDa protein in the absence of urea. Under the same elec-

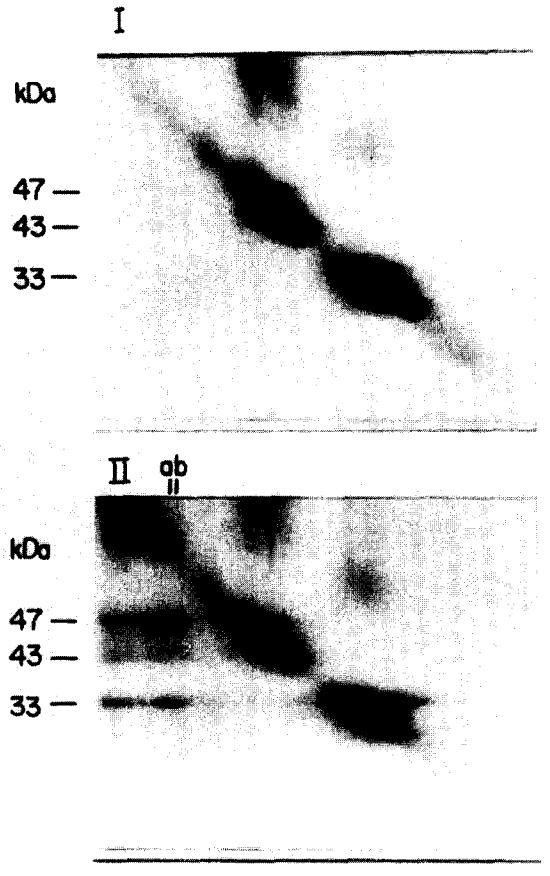

Fig.3. Diagonal electrophoresis of the oxygen-evolving complexes. Gels contained no urea. Acrylamide concentration was 11.5% in both the first- and second-dimensional electrophoreses. I, untreated complexes; II, complexes treated with 0.02% DSP.

trophoretic conditions, the complexes, which had been crosslinked with DSP and then treated with 2-mercaptoethanol, yielded the 47 and 33 kDa off-diagonal spots (fig.3II). Long tailings of the spots are due to poor resolution of the crosslinked products in the one-dimensional electrophoresis in gels containing no urea. The results clearly show that the 47 kDa spot is derived from the crosslinked products but not from the dimer of the 47 kDa protein. We concluded therefore that the bands a and b are both crosslinked products of the 47 and 33 kDa proteins.

Then the question arises as to why crosslinking of the 47 and 33 kDa proteins gives rise to two split product bands a and b. When excised from the one-dimensional gels and reelectrophoresed after cleavage of the crosslinker, the bands a and b yielded identical protein patterns (not shown). Thus, different electrophoretic mobilities of the two bands are ascribed to difference in neither composition nor stoichiometry of the crosslinked proteins. We suggest therefore that the two bands are different in degree of intramolecular crosslinking, which would affect of the bulk conformation of the crosslinked products.

4. CONCLUDING REMARKS

The present work shows that DSP, a cleavable bifunctional reagent with a maximum crosslinking span of 1.2 nm [13], specifically crosslinks the 33 kDa extrinsic protein and the 47 kDa chlorophyll-carrying protein of the PS II reaction center complexes. It is likely therefore that the 33 kDa protein is attached to the oxygen-evolving complexes by binding to a domain of the 47 kDa protein exposed to the lumenal phase.

Manganese atoms are assumed to be located at, or near the interface between the 33 kDa protein and (a) subunit(s) of the PS II reaction center complex [16,17]. Thus, the results obtained here seem to suggest that the 47 kDa protein carries the Mn-binding sites. However, this view awaits further confirmation by other approaches because failure to detect a crosslinked product of the 33 kDa protein with other subunit proteins of the PS II reaction center complexes does not necessarily mean that the 33 kDa protein is associated with, or located near, only the 47 kDa subunit. A possibility remains that the 33 kDa protein binds to the D-1 or D-2 protein, or the 43 kDa protein of the core complex. Isogai et al. [18] have suggested association of the 33 kDa protein with the 43 kDa protein because the removal of the 33 kDa protein enhanced proteolytic digestion of the 43 kDa protein.

Finally, the present work shows that chemical crosslinking with a cleavable reagent is a useful tool to analyze mutual arrangement of subunit proteins in the PS II oxygen-evolving complex. It is important to use a complex with as simple a subunit composition as possible because the smaller the number of crosslinked products, the easier the identification of crosslinked proteins.

ACKNOWLEDGEMENTS

The present work was supported in part by grants from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Åkerlund, H.-E. and Jansson, C. (1981) FEBS Lett. 124, 229–232.
[2] Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268.
[3] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[4] Toyoshima, Y. and Fukutaka, E. (1982) FEBS Lett. 150, 223–227.
[5] Murata, N., Miyao, M. and Kuwabara, T. (1983) in: The Oxygen Evolving System of Photosynthesis (Inoue, Y. et al. eds) pp.213–222, Academic Press, Tokyo.
[6] Ono, T. and Inoue, Y. (1983) FEBS Lett. 164, 255–260.
[7] Tang, X.-S. and Satoh, K. (1985) FEBS Lett. 179, 60–64.
[8] Satoh, K., Ohno, T. and Katoh, S. (1985) FEBS Lett. 180, 326–330.
[9] Ikeuchi, M., Yuasa, M. and Inoue, Y. (1985) FEBS Lett. 185, 316–322.
[10] Satoh, K. (1985) Photochem. Photobiol. 42, 845–853.
[11] Enami, I., Miyaoka, T., Igarashi, S., Satoh, K. and Katoh, S. (1986) in: Progress in Photosynthesis Research (Biggins, J. ed.) vol.1, pp.709–712, Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Middaugh, C.R., Vanin, E.F. and Ji, T.H. (1983) Mol. Cell. Biochem. 50, 115–141.
[14] Millner, P. and Barber, J. (1985) Physiol. Vég. 23, 767–775.
[15] Adir, N. and Ohad, I. (1986) Biochim. Biophys. Acta 850, 264–274.
[16] Ono, T. and Inoue, Y. (1984) FEBS Lett. 168, 281–286.
[17] Yamamoto, Y., Shinkai, H., Isogai, Y. and Nishimura, M. (1984) FEBS Lett. 175, 429–432.
[18] Isogai, Y., Yamamoto, Y. and Nishimura, M. (1985) FEBS Lett. 187, 240–244.