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Chemical crosslinking studies of extrinsic proteins in cyanobacterial photosystem II

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Abstract Chemical crosslinking with a zero-length crosslinker, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride, was applied to a cyanobacterial photosystem II complex retaining three extrinsic proteins, the 33 kDa manganese-stabilizing protein, cytochrome (cyt) *c*-550 and the 12 kDa protein. Three major crosslinked products were obtained in addition to the crosslinked product between the extrinsic 33 kDa and the intrinsic CP47 proteins. They were identified to be: cyt *c*-550–12 kDa; cyt *c*-550–12 kDa–33 kDa; D2–cyt *c*-550–12 kDa. These results indicate that the three extrinsic proteins are closely located with each other in cyanobacterial PSII, supporting the previous proposal that, like the 33 kDa protein, cyt *c*-550 and the 12 kDa protein are associated with PSII at the lumenal side of thylakoids. The results also suggested that the D2 reaction center protein provides a direct binding site for the extrinsic cyt *c*-550.

Key words: Cytochrome *c*-550; Photosystem II; Extrinsic protein; O_2 evolution; Crosslinking; *Synechococcus*

1. Introduction

Cyanobacterial PSII contains the well characterized extrinsic protein of 33 kDa that stabilizes binding of the Mn-cluster and thereby maintains oxygen-evolving activity (for review see [1]). In addition to this protein, Shen et al. [2] have recently identified two more new extrinsic components, cyt *c*-550 and a 12 kDa protein, in a PSII complex purified from a thermophilic cyanobacterium, *Synechococcus vulcanus*. These two extrinsic proteins bind to PSII stoichiometrically and are released, together with the 33 kDa protein, by washing with high concentrations of $CaCl_2$ or alkaline Tris. Absence of cyt *c*-550 and the 12 kDa protein resulted in a partial loss of oxygen evolution, which was reversed by rebinding of the two proteins [3]. Release-reconstitution experiments also revealed that, while cyt *c*-550 can bind to PSII in a manner essentially independent of the other two proteins, the 12 kDa protein can bind to PSII functionally only when both the 33 kDa protein and cyt *c*-550 have been already bound [3]. These results suggested that the three extrinsic proteins are in close contact with each other. Since the 33 kDa protein is known to bind to PSII at the lumenal side of thylakoids, this in turn suggests that the newly identified cyt *c*-550 and 12 kDa protein are also associated with PSII at the lumenal side of thylakoids.

In order to determine the nearest neighbor relationships among the extrinsic proteins and also between extrinsic and intrinsic components in cyanobacterial PSII, we carried out chemical crosslinking studies with purified *S. vulcanus* PSII using a zero-length crosslinker, EDC. The results revealed that the three extrinsic proteins are indeed in close contact with each other, since a crosslinked product containing all the three extrinsic proteins was obtained. It was also shown that cyt *c*-550 crosslinks with both the 12 kDa protein and the D2 protein, suggesting a direct association of this cytochrome to the reaction center. Part of the results have been reported at a preliminary stage in [4].

2. Materials and methods

Cyanobacterial PSII particles were purified from thermophilic *Synechococcus vulcanus* as described in [3,5] and suspended in 40 mM MES (pH 6.0), 50 mM NaCl. Cyt *c*-550 was purified from $CaCl_2$-extracts of the PSII particles by column chromatography [3]. Crosslinking with various concentrations of EDC was carried out at a chlorophyll concentration of 1 mg per ml for 10 min in the dark at room temperature. The crosslinking reaction was terminated by the addition of sodium acetate (final concentration, 0.25 M) as described by Enami et al. [6].

For protein analysis, crosslinked PSII samples were treated with 10% LDS and 60 mM dithiothreitol in 0.37 M Tris (pH 8.5) for 30 min at room temperature, and then subjected to SDS-PAGE on a gel containing 16% acrylamide and 7.5 M urea. After electrophoresis, gels were either stained with CBB or transferred to nitrocellulose membranes (0.2 mm in thickness, Schleicher and Schuell) for immunoblotting. Immunoblot reactions were detected by alkaline phosphatase-linked immunoblot analysis. Antibodies against *S. vulcanus* cyt *c*-550 and the 12 kDa proteins were constructed as described previously [5]. Antibodies against PSII D1, D2, 47 kDa, 43 kDa and 33 kDa proteins were raised against respective spinach proteins.

3. Results

Fig. 1 shows CBB-stained polypeptide profiles of the thermophilic cyanobacterial PSII after treatment with various concentrations of EDC. With increase in EDC concentration, the staining intensity of the 12 kDa protein band decreased and finally disappeared at EDC concentrations above 0.4%, implying that this protein was efficiently crosslinked by EDC to form a complex of higher molecular masses. Band intensities of the

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Abbreviations: CBB, Coomassie brilliant blue R-250; CP47 and CP43, 47 kDa and 43 kDa chlorophyll-binding core antenna proteins of photosystem II, respectively; cyt, cytochrome; D1 and D2, 30 and 32 kDa reaction center proteins of photosystem II, respectively; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride; MES, 4-morpholine-ethanesulphonic acid; PSII, photosystem II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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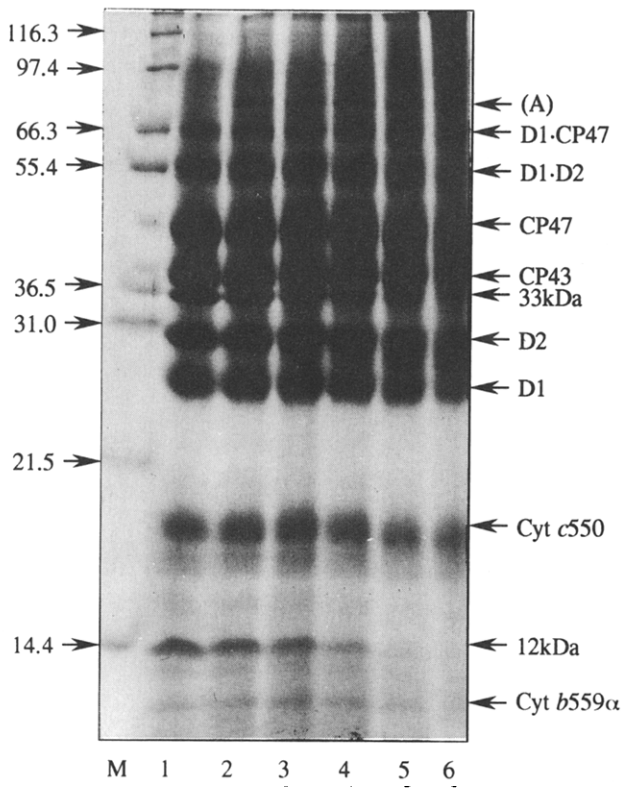

Fig. 1. CBB-stained SDS-PAGE profiles of *S. vulcanus* PSII complexes after treatment with various concentrations of EDC. The EDC concentrations were: Lane 1, 0%; lane 2, 0.1%; lane 3, 0.2%; lane 4, 0.4%; and lane 5, 0.8%. Molecular markers used are: 116.3 kDa, β-galactosidase; 97.4 kDa, phosphrylase b; 66.3 kDa, bovine serum albumin; 55.4 kDa, glutamic dehydrogenase; 36.5 kDa, lactate dehydrogenase; 31.0 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor; 14.4 kDa, lysozyme.

extrinsic 33 kDa protein and cyt *c*-550 showed, although in a smaller degree, a similar decrease upon increase in EDC concentration, suggesting that these extrinsic proteins are also crosslinked by EDC. As to the products of the crosslinking reaction, however, only one band of about 75 kDa (indicated by band A in Fig. 1) could be detected clearly on the CBB-stained gel, the other possible crosslinked products could not be detected probably due to their co-migration with intrinsic PSII components or otherwise, they are not enough in quantity to be visible by CBB-staining.

In order to identify the crosslinked products, we employed immunoblot analysis with antibodies raised against the major PSII components and those against the three extrinsic proteins as well. Fig. 2 summarizes the results of our immunoblot analysis. The 75 kDa band visible in CBB-stained gel (Band A in Fig. 1) after EDC-treatment showed immuno-reactivity with antibodies against the 33 and 47 kDa proteins (indicated by solid circles in panels C and D of Fig. 2), indicating that the 75 kDa band is a crosslinked product of the 33 and 47 kDa proteins. This result is consistent with those obtained with higher plant PSII, where the 33 and 47 kDa proteins were efficiently crosslinked by EDC [7,8]. The band at 45 kDa that appeared after EDC-treatment as indicated by open triangles in panels A, B and C of Fig. 2, showed immuno-reactivity with antibodies against D2, cyt *c*-550 and the 12 kDa protein. This implies that the 45 kDa band is either a mixture of crosslinked products between different components, such as D2-*c*-550 and D2–12 kDa protein, or it is a crosslinked product of these three proteins. Since the 45 kDa band appeared quite sharp on the gel, it is unlikely that it contains multiple products of the crosslinking reaction. In other words, the 45 kDa band is most likely a crosslinked product of D2, cyt *c*-550 and the 12 kDa protein. The apparent molecular mass of this band was slightly smaller than 60 kDa that would be expected from the sum of the molecular masses of the three proteins, probably due to some conformational modifications upon EDC crosslinking. The same situation could be pointed out for another crosslinked product of 50 kDa that was found to react with antibodies against the three extrinsic proteins, 33 kDa, cyt *c*-550 and the 12 kDa proteins (indicated by solid triangles in panels B, C and D in Fig. 2). The total molecular mass of these three extrinsic proteins is 62 kDa based on their respective apparent molecular masses determined by SDS-PAGE. The band of 27 kDa indicated by open circles in panels B and C showed immunoreactivity with antibodies against cyt *c*-550 and 12 kDa protein, indicating a crosslinkage of cyt *c*-550 with the 12 kDa protein. The weak band slightly below the major band of cyt *c*-550 (panel B) is probably a degradation product of the cyt [3]

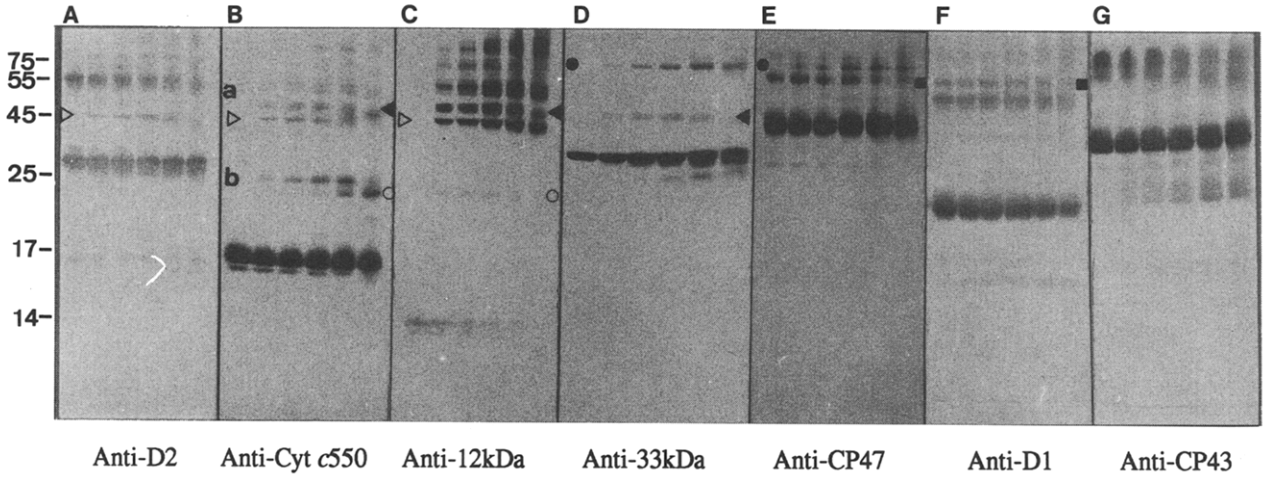

Fig. 2. Immunoblot analysis of EDC-treated PSII complexes probed with antibodies against D2 (A); cyt *c*-550 (B); extrinsic 12 kDa protein (C); extrinsic 33 kDa protein (D); CP47 (E); D1 (F) and CP43 (G). Lanes from left to right in each panel correspond the EDC concentrations indicated in Fig. 1.

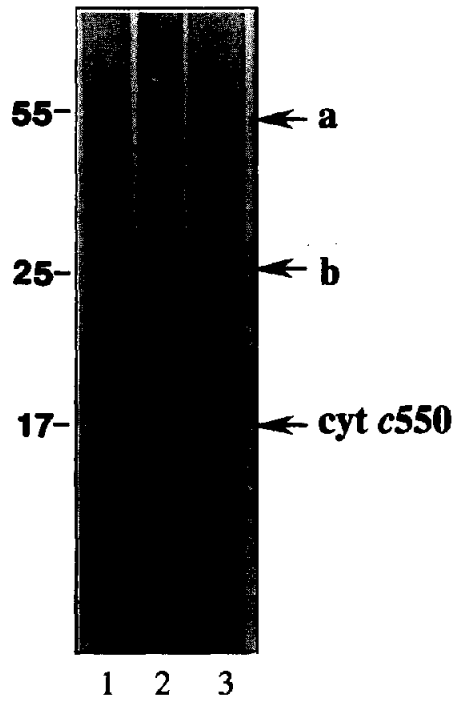

Fig. 3. Immunoblot analysis with anti-cyt *c*-550 of EDC crosslinking of purified cyt *c*-550. Lane 1: EDC-untreated PSII; lane 2: EDC-crosslinked PS II (equivalent to lane 5 in panel B of Fig. 2); lane 3: EDC-crosslinked purified cyt *c*-550.

(see also Fig. 3). In addition, a band could be detected at 70 kDa in untreated PSII (solid squares in panels E and F). This band was found to cross-react with antibodies against CP47 and D1 protein in PSII even prior to the EDC-treatment, indicating that this was not a crosslinked product but a CP47-D1 heterodimer that was spontaneously formed during SDS-PAGE procedure or existed in our original PSII preparation. This CP47-D1 heterodimer perhaps is a feature for purified cyanobacterial PSII since it has not been reported with higher plant PSII. Nonetheless, the presence of this heterodimer did not interfere with the identification of other crosslinked products, since none of the crosslinked products involved both CP47 and D1. A similar situation was seen for the well known D1-D2 hetrodimer that showed immuno-reactivity with antibodies against D1 and D2 in EDC-untreated PSII (see panels A and F).

In Fig. 2, we also recognized two faint bands at 25 and 55 kDa (bands a and b in panel B), which showed immuno-reactivity with antibody against cyt *c*-550 but apparently not with any other antibodies. This points to the possibility that they were intermolecular crosslinked products of cyt *c*-550. To check this possibility, the same EDC crosslinking experiment was carried out with purified cyt *c*-550. As Fig. 3 shows, EDC-treatment of purified cyt *c*-550 indeed gave rise to two bands at the same position as those obtained with EDC-treatment of PSII complex. This clearly indicates that the two bands are really intermolecular crosslinked cyt *c*-550. Based on their apparent molecular masses, the 25 kDa band is probably a crosslinked product consisting of two molecules of cyt *c*-550, whereas the 55 kDa band probably contains three to four molecules of the cyt. The intermolecular crosslinking of cyt *c*-550 even when it is associated with PSII implies that either the isolated PSII complexes were present in dimeric or even multimeric form or the cyt is easy to be crosslinked intermolecularly between different PSII complexes due to its association with the surface of PSII. In Fig. 2, we also recognized several dense bands in higher molecular mass region after EDC-treatment. These bands cross-reacted only with antibody against the 12 kDa protein, showing no other apparent counterparts detectable by immunological analysis. Thus, these are probably all intermolecular crosslinked products of the extrinsic 12 kDa protein consisting of different numbers of the protein molecule. It should be mentioned here that, the staining intensities of the presumable intermolecular crosslinked products of the 12 kDa protein (high molecular weight bands in lanes 2–6 of Fig. 2C) is much higher than the staining intensity of the non-crosslinked 12 kDa protein (lane 1 of fig. 2C). This may be due to a blotting through the nitrocellulose membrane of the non-crosslinked protein, which occurs more easily for low molecular weight proteins.

4. Discussion

One major crosslinked product obtained upon treatment of the thermophilic cyanobacterial PSII with EDC involves the three extrinsic proteins of 33 kDa, cyt *c*-550 and 12 kDa. In addition, a crosslinked product containing cyt *c*-550 and the 12 kDa protein was also observed. These results indicate that the three extrinsic proteins are located closely with each other, thus implying that, like the extrinsic 33 kDa protein, cyt *c*-550 and the 12 kDa protein are also located on the lumenal side of PSII. This agrees with and further supports the results of previous release-reconstitution experiments that the three extrinsic proteins are in close contact with each other in terms of their binding [3]. Crosslinkage of the 12 kDa protein with cyt *c*-550 and the 33 kDa protein is also consistent with the idea that the later two extrinsic proteins provide a binding site(s) for the 12 kDa protein, which has been previously proposed based on the fact that the 12 kDa protein cannot bind to PSII unless the other two proteins are already present [3].

Another major crosslinked product obtained contained the D2 reaction center protein and cyt *c*-550, 12 kDa protein. This suggests that cyt *c*-550 and the12 kDa protein are in close contact with the D2 protein. Because the 12 kDa protein cannot bind to PSII in the absence of cyt *c*-550 and the 33 kDa protein, and also because cyt *c*-550 and 12 kDa protein can be crosslinked by EDC, we may conclude that cyt *c*-550, but not the 12 kDa protein, is directly crosslinked with D2. Since cyt *c*-550 can bind to cyanobacterial PSII independently [3], this may suggest that the intrinsic D2 protein provides a binding site for cyt *c*-550.

The present report provides a first chemical crosslinking study on cyanobacterial PSII, although many such studies have been conducted on higher plant PSII. Consistent with the results obtained from higher plant PSII [7,8], the extrinsic 33 kDa protein is crosslinked with CP47 in cyanobacterial PSII. There are, however, some apparent differences between cyanobacterial and higher plant PSII in the crosslinking pattern of the other two extrinsic proteins, cyt *c*-550 and 12 kDa protein in cyanobacteria and 23, 17 kDa proteins in higher plants, although these two sets of proteins share some common features in their binding and function. Cyt *c*-550 crosslinks directly with the 33 kDa protein with the zero-length crosslinker, EDC, in cyanobacterial PSII, whereas in higher plants the 23 kDa protein crosslinks with the 33 kDa protein only with crosslink-

ers of 6–12 Å in length [9]. In addition, cyt *c*-550 directly crosslinks with D2 but the 23 kDa protein has not been reported to crosslink with any intrinsic proteins in higher plant PSII. This may reflect the situation that cyt *c*-550 can directly bind to PSII while the 23 kDa protein can bind to PSII only in the presence of the 33 kDa protein [10]. In contrast to these, the 12 kDa protein only crosslinks with the extrinsic cyt *c*-550 and possibly also with the 33 kDa protein, but not with any PSII intrinsic proteins. This is consistent with the fact that the 12 kDa protein can bind to PSII only in the presence of the 33 kDa protein and cyt *c*-550. This situation is similar to that reported for the 17 kDa protein in higher plant PSII, where the 17 kDa protein crosslinks with the 23 kDa protein but not with other intrinsic proteins [9]. The 17 kDa protein is also incapable of binding to PSII without the 33 and 23 kDa proteins [10,11]. These similarities strongly suggest that the extrinsic 12 kDa protein in cyanobacterial PSII resembles the 17 kDa protein in higher plant PSII with respect to its binding to PSII.

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