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Close association of the 33 kDa extrinsic protein with the apoprotein of CPa1 in photosystem II

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The structural arrangement of the extrinsic 33 kDa protein and the 49/53 kDa apoprotein of CPa1 were investigated in oxygen-evolving photosystem II preparations. *N*-Hydrosuccinimidobiotin (NHS-biotin) was used to label accessible amino groups in control, NaCl-, $CaCl_2$- and alkaline tris-washed membranes. Labeling of the apoprotein of CPa1 was observed in treatments which removed the extrinsic 33 kDa protein. The water-soluble carbodiimide, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) was used to crosslink proteins with complementary charged groups in close proximity to one another. Two crosslinked complexes of the extrinsic 33 kDa and the CPa1 apoprotein were observed at 76 and 65 kDa. These complexes were not formed in membranes lacking the 33 kDa extrinsic protein. Finally, the homobifunctional cleavable crosslinker, dithio*bis*[succinimidylpropionate] (DTSP), was used to reversibly crosslink the 33 kDa extrinsic protein with the apoprotein of CPa1 in oxygen-evolving PS II core complex preparations. These results suggest a very close association of the extrinsic 33 kDa protein and the apoprotein of CPa1 in the photosynthetic membrane. We suggest that the apoprotein of CPa1 may provide a binding site for the 33 kDa extrinsic component.

Photosystem II; CPa1; 33 kDa protein; Protein crosslinking; NHS-biotin labeling

1. INTRODUCTION

Recently, both the structure and function of PS II have been under intensive investigation. Polypeptides of 49, 45, 34 (D-1), 33, 32 (D-2) and 9 kDa (cytochrome *b*-559) in association with bound manganese appear to form the minimum complex capable of photosynthetic oxygen evolution [1]. The D-1, D-2 and cytochrome *b*-559 subunits form an isolatable core of PS II [2]. While not capable of oxidizing water, this core can carry out a number of partial reactions associated with the photosystem [2,3]. The 49 and 45 kDa polypeptides are the apoproteins of CPa1 and CPa2, respectively [4]. These chlorophyll proteins act as interior chlorophyll *a* antennae for PS II. Additional roles for these proteins, such as manganese-binding or possible binding sites for the extrinsic proteins associated with PS II, cannot be excluded at this time given the experimental evidence available. The 33 kDa extrinsic protein is required for high rates of oxygen evolution at physiological salt concentrations and appears to stabilize the bound manganese against loss in low ionic strength environments [5].

Unfortunately, relatively little is known of the structural arrangement of these polypeptides within the PS II complex. Since no crystal structure is yet available for higher plant PS II, investigators have had to rely on indirect methods of elucidating structural associations among these polypeptides. The majority of these efforts have centered on the relationship between the extrinsic

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Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; DTSP, dithio*bis*[succinimidylpropionate]; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; LDS, lithium dodecyl sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NHS-biotin, *N*-hydrosuccinimidobiotin; PAGE, polyacrylamide gel electrophoresis; PS II, photosystem II; SADP, *N*-succinimidyl-[(4-azidophenyl)dithio]propionate; Tris, tris(hydroxymethyl)-aminomethane

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33 kDa polypeptide and the intrinsic components of PS II. In oxygen-evolving PS II membrane preparations, the extrinsic 33 kDa polypeptide apparently shields a number of PS II components from tryptic attack including the 49 kDa [6] and the 45 kDa [7] apoproteins of CPa1 and CPa2, respectively. Removal of the 33 kDa extrinsic polypeptide from PS II membranes greatly increases the ability of certain peptide-specific polyclonal antibodies to bind to the D-1 protein [8]. Additionally, the removal of this extrinsic component and bound manganese from PS II membranes dramatically increases the binding of a monoclonal antibody to the 49 kDa protein [6]. Apparently, the removal of bound manganese induces a conformational change in either the 49 kDa polypeptide or a protein component closely associated with the 49 kDa polypeptide which increases the exposure of the antigenic determinant recognized by the monoclonal antibody. This antigenic determinant resides on a 14.5 kDa cyanogen bromide fragment of the 49 kDa protein [6].

Bowlby and Frasch [9] have isolated the 33 kDa extrinsic protein, modified it with the crosslinking agent SADP and reconstituted $CaCl_2$-washed PS II membrane preparations with the modified protein. A crosslinked complex was then obtained after detergent treatment which contained the 33 kDa extrinsic protein crosslinked by SADP to a number of other components in the 20–25 kDa molecular mass range and possibly bound manganese. Ljungberg et al. [10] used specific polyclonal antibodies against the 33 kDa extrinsic protein to co-precipitate peptides of 24 and 22, as well as a 10 kDa polypeptide which is distinct from cytochrome *b*-559. It should be pointed out that the proteins isolated in association with the extrinsic 33 kDa protein in these studies are not present in oxygen-evolving core preparations, and therefore, are not directly involved in oxygen evolution. They may, however, play some role in stabilizing the 33 kDa extrinsic protein in the native membrane environment.

In this communication, we present additional evidence for the close association of the 33 kDa extrinsic protein with the 49 kDa apoprotein of CPa1. This evidence is two-fold and includes the specific labeling of the CPa1 apoprotein with NHS-biotin in the absence of the 33 kDa extrinsic protein, as well as the crosslinking of the 33 kDa extrinsic protein to the 49 kDa protein by both the water-soluble carbodiimide, EDC, and the cleavable crosslinker, DTSP.

2. MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as described [11]. Chl was determined by the method of Arnon [12]. Oxygen-evolving PS II membranes were prepared essentially by the procedure of Ghanotakis and Babcock [13], with the modifications noted in [14]. Oxygen-evolving PS II reaction center core complex was prepared essentially as described by Ghanotakis et al. [1]. NaCl, $CaCl_2$ and alkaline-tris treatments, which remove the extrinsic polypeptides and the bound manganese from the PS II oxygen-evolving membranes, were performed as in [6] with the exception that, in the EDC crosslinking experiments, the membranes were finally rinsed with, and resuspended in, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl and 50 mM Na-K phosphate buffer, pH 6.0.

For the NHS-biotin labeling experiments, control and treated PS II oxygen-evolving membranes were suspended at 1.0 mg/ml Chl in 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl and 50 mM Mes-NaOH, pH 6.0 (resuspension buffer). NHS-biotin was dissolved at a concentration of 1.0 mg/ml in dimethyl sulfoxide. 20 μl of this NHS-biotin solution was added to 1.0 ml of the different membrane suspensions and allowed to incubate for 1 h at 0–4°C. At the end of the incubation, the membranes were washed three times with, and resuspended in, resuspension buffer. The labeled membranes were then analyzed by LDS-PAGE. Samples were solubilized in 2% LDS, 6% sucrose, 5% β-mercaptoethanol and a trace of bromophenol blue, at 0–4°C. Electrophoresis by the procedure of Delepelaire and Chua [4] was performed in 12.5–20% acrylamide linear gradient gels at 1 W for 20 h at 4°C. After electrophoresis, the separated proteins were electrotransferred to nitrocellulose paper by the method of Towbin et al. [15]. One half of this Western blot was stained with amido black, while the other half was blocked with 1% non-fat dry milk in Tris-saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 4 h at room temperature. After blocking, the Western blot was probed with avidin-peroxidase conjugate (500 ng/ml in Tris-saline containing 1% BSA) for 4 h at 22°C. The blot was then washed extensively with Tris-saline. Biotinylated proteins were visualized by incubation of the Western blot with 4-chloro-1-naphthol [16] and H_2O_2.

For EDC crosslinking, control and treated PS II membrane preparations were suspended at 1.0 mg/ml Chl in 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, 50 mM Na-K phosphate buffer, pH 6.0. These membranes were then incubated with a range of EDC concentrations (0–25 mM) for 20 min at 0–4°C. The modified PS II membranes were washed twice with resuspension buffer and were stored at −20°C prior to LDS-PAGE. Electrophoresis was performed as described above. Additionally, the samples were heated at 70°C for 5 min prior to electrophoresis. The separated proteins were blotted to nitrocellulose and the Western blots blocked as described above. These blots were then incubated overnight with either the murine monoclonal antibody, FAC2 [6], which recognizes

the 49 kDa apoprotein of CPa1, or with α33, a murine polyclonal antibody raised against purified 33 kDa extrinsic protein [17]. After washing with Tris-saline, the blots were incubated for 4 h with a 1:2000 dilution of goat anti-mouse IgG IgM-peroxidase conjugate in Tris-saline containing 1% BSA, washed with Tris-saline and developed with 4-chloro-1-naphthol as described above.

For DTSP crosslinking, oxygen-evolving PS II reaction center core complex [1] was resuspended to a Chl concentration of 1.0 mg/ml in 50 mM Na-K phosphate buffer, pH 7.0. DTSP was added from a 50 mM stock solution (freshly prepared for each experiment) in dimethyl sulfoxide to a final concentration of 500 μM. After incubation at 0–4°C for 100 min, the crosslinking reaction was terminated by bringing the reaction mixture to 20 mM glycinamide and incubating for 30 min. Disulfide exchange reactions were inhibited by the addition of iodoacetamide to a final concentration of 40 mM and incubating for 45 min. Samples were stored at −20°C prior to electrophoresis. Preliminary experiments established that no crosslinked products were observed in the presence of dimethyl sulfoxide, glycinamide and/or iodoacetamide, in the absence of DTSP.

The proteins crosslinked by DTSP were resolved by two-dimensional diagonal electrophoresis followed by Western blotting and immunological detection. In the first dimension, the crosslinked proteins were separated by SDS-PAGE in the absence of reducing agents. This electrophoresis was performed in 12.5–20% acrylamide linear gradient tube gels. The buffers employed by Delepelaire and Chua [4] were used with the exception that SDS was substituted for LDS. Electrophoresis was performed at 40 mW/tube gel for 12 h at 4°C. Immediately following the first dimension electrophoresis, the gel was equilibrated for 20 min at 22°C in a buffer consisting of 125 mM Tris-HCl, pH 6.8, 1.33% SDS, 5% β-mercaptoethanol and a trace amount of bromophenol blue. The equilibrated first dimension gel was then layered onto a second dimension SDS-polyacrylamide slab gel (12.5–20% acrylamide linear gradient), sealed in place with 0.5% agarose containing 125 mM Tris-HCl, pH 6.8, and electrophoresed for 24 h at 1.5 W at 4°C. The upper reservoir buffer contained 0.02% thioglycolic acid, in addition to the usual buffer components [4], to maintain reducing conditions during electrophoresis. After electrophoresis, the gels were electroblotted and blocked as described above. The Western blot was then probed simultaneously with both FAC2 and α33, incubated with goat anti-mouse IgG + IgM-peroxidase conjugate and developed with 4-chloro-1-naphthol as described.

3. RESULTS AND DISCUSSION

The results of NHS-biotin labeling of control (unwashed) and treated PS II membranes are shown on the Western blot depicted in fig.1. Lane 1 illustrates that, in the absence of NHS-biotin, none of the proteins present in PS II membranes bind the avidin-peroxidase conjugate. Under the mild electrophoretic solubilization conditions employed, LHC II migrates as a green, chlorophyll-protein and, thus, is visible on these photographs. In unwashed, NHS-biotin-labeled PS II membranes (lane 2), a number of proteins do react with the avidin-peroxidase conjugate, indicating their biotinylation. These include the three extrinsic proteins associated with the oxygen-evolving site at apparent molecular masses of 33, 24, and 17 kDa. Additionally, polypeptides in the 22–28 kDa region are biotinylated. Only a trace amount of labeling can be seen in the 49/53 kDa component, which is the apoprotein of CPa1 [6]. After washing with NaCl (lane 3) a similar pattern of labeling is observed with the exception that the 24 and 17 kDa extrinsic polypeptides have been removed. Again, only a slight amount of labeling is observed in the apoprotein of CPa1. After $CaCl_2$ or alkaline-tris washing (lanes 4 and 5, respectively), however, a marked change in the labeling pattern is observed. The 33 kDa extrinsic component is removed by these treatments. Additionally, the CPa1 apoprotein is now heavily labeled. These results indicate that, in the absence of the extrinsic 33 kDa polypeptide, free amino groups present on the CPa1 apoprotein become accessible to NHS-biotin. Since NHS-biotin is a small probe (341 kDa) the extrinsic 33 kDa polypeptide is either physically shielding these free amino groups, or, loss of the extrinsic 33 kDa protein induces a conformational change in the CPa1 apoprotein (or some other closely associated protein), which exposes these amino groups for labeling. These hypotheses suggest a very close association between the extrinsic 33 kDa and the intrinsic 49/53 kDa apoprotein of CPa1.

The water-soluble carbodiimide EDC has been a very useful reagent for the crosslinking of proteins which interact by charged-paired mechanisms [18]. In control and NaCl-washed PS II membranes, treatment with EDC generates crosslinked products with apparent molecular masses of 76 and 65 kDa which crossreact with both the monoclonal antibody FAC2 (fig.2A) and the polyclonal antibody α33 (fig.2B). Increasing amounts of these crosslinked products form with increasing EDC concentrations. Neither of these crosslinked products were detected by these antibodies when $CaCl_2$- or alkaline-tris-washed membranes were treated with EDC (fig.2A and B). These results strongly suggest that the 76 and 65 kDa crosslinked products consist minimally of

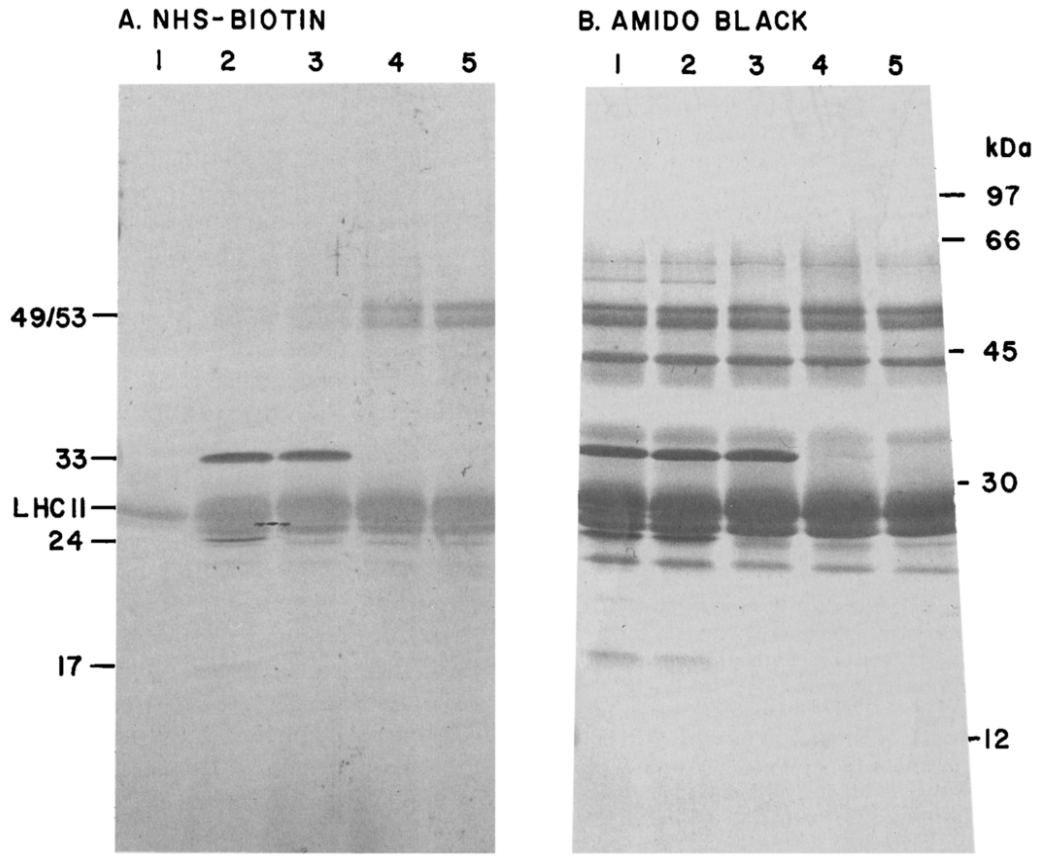

Fig.1. NHS-biotin labeling of control and polypeptide-extracted PS II membranes. NHS-biotin was used to label differentially washed PS II membrane preparations. After treatment, the proteins were separated by LDS-PAGE and transferred to nitrocellulose paper. (A) This panel was blocked and probed with an avidin-peroxidase conjugate. Color development at the location of biotinylated proteins was observed after incubation with 4-chloro-1-naphthol and H_2O_2. (B) This panel was stained with amido black immediately after transfer to nitrocellulose paper. Lanes: 1, unlabeled control membranes; 2, NHS-biotinylated control membranes; 3, NHS-biotinylated NaCl-washed membranes; 4, NHS-biotinylated $CaCl_2$-washed membranes; 5, NHS-biotinylated alkaline-tris-washed membranes. The location of molecular mass standards are shown on the right. Pertinent PS II components are identified on the left.

the apoprotein of CPa1 and the 33 kDa extrinsic polypeptide. The possible presence of other components in these crosslinked complexes cannot be excluded at this time. The apparent molecular masses of the crosslinked complexes, however, are most consistent with the CPa1 apoprotein and the 33 kDa extrinsic polypeptide being present at a 1:1 stoichiometry. It should be noted that these samples were heated at 70°C for 5 min prior to electrophoresis. This treatment converts most of the 49/53 kDa polypeptide to a more rapidly migrating, 44 kDa form, as has been described [6]. In addition to the 76 and 65 kDa crosslinked complexes, three other bands which crossreact with α33 are present in fig.2B. The polypeptide composition of the crosslinked products with apparent molecular masses of 57 and 37 kDa, have not been determined at this time. It is highly probable that the 26 kDa crosslinked product represents an intramolecular crosslinking event.

Several additional features of fig.2 should be noted. First, only relatively small amounts of the 76 and 65 kDa crosslinked products are detected with either FAC2 or α33. The primary reason for this is the necessity to use relatively low concentrations of EDC to minimize formation of intercomplex crosslinked products. EDC concentrations are chosen which will crosslink only

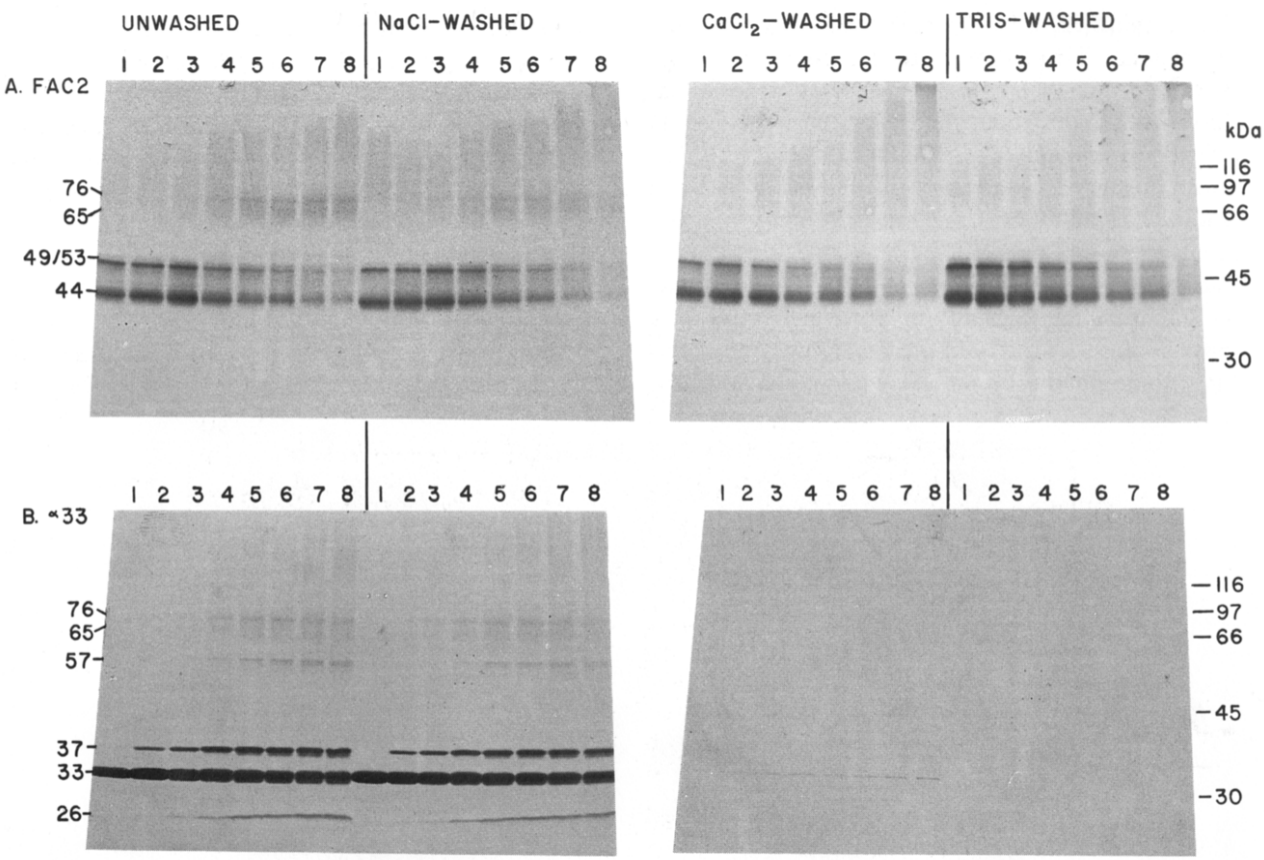

Fig.2. EDC crosslinking of the 49/53 kDa apoprotein of CPa1 and the 33 kDa extrinsic polypeptide. Unwashed (control), NaCl-washed, $CaCl_2$-washed and alkaline-tris-washed membranes were treated with EDC at various concentrations, their proteins separated by LDS-PAGE and then transferred to nitrocellulose paper. After blocking, these Western blots were probed with: (A) the monoclonal antibody FAC2, which recognizes the apoprotein of CPa1; or (B) α33, a polyclonal antibody which recognizes the extrinsic 33 kDa protein. Color development was observed after incubation with goat anti-mouse IgG + IgM-peroxidase conjugate, followed by incubation with 4-chloro-1-naphthol and H_2O_2. Lanes: 1, 0 mM EDC; 2, 1.0 mM EDC; 3, 2.0 mM EDC; 4, 5 mM EDC; 5, 10 mM EDC; 6, 15 mM EDC; 7, 20 mM EDC; 8, 25 mM EDC. The location of molecular mass standards are shown on the right. Crosslinked products and the parent polypeptides are identified on the left.

5–10% of the total antibody-detectable protein present. Secondly, there is a general loss of labeling intensity of the uncrosslinked apoprotein of CPa1 in fig.2A which is not observed with the uncrosslinked 33 kDa protein in fig.2B. This loss of labeling intensity is not dependent upon the formation of the 76 and 65 kDa crosslinked complexes since it is present in the $CaCl_2$- and alkaline-tris-washed lanes of fig.2A. This labeling loss is, however, dependent on concentration of EDC. Concomitant to the loss of uncrosslinked labeling is the appearance of a broad, indistinct background staining with increased EDC concentrations. These phenomena may be related, and may suggest crosslinking of the apoprotein of CPa1 with other membrane components whose identity has not yet been determined. Finally, it should be noted that no quantitative speculations comparing fig.2A and B are warranted. Since we are comparing two different antigens with two different antibodies no quantitative conclusions can be reached.

DTSP is a homobifunctional cleavable protein crosslinking reagent which has been widely used [19]. Oxygen-evolving reaction center core complex [1] was treated with DTSP and analyzed by diagonal two-dimensional electrophoresis. Proteins which were intermolecularly crosslinked by

DTSP migrated as a vertical series of spots below a diagonal formed by non-crosslinked proteins. Proteins which were intramolecularly crosslinked lie above this diagonal. Fig.3 illustrates the results of this experiment. Proteins which react with the antibodies FAC2 and α33 form a series of vertical spots lying below the diagonal indicating that the apoprotein of CPa1 and the 33 kDa extrinsic protein had been crosslinked by DTSP. The crosslinked complex migrates at an apparent molecular mass of about 101 kDa in the first dimension. This high apparent molecular mass suggests that proteins other than the 33 kDa protein and the apoprotein of CPa1 may be present in the crosslinked complex. Preliminary evidence suggests that one of these proteins may be cytochrome *b*-559 (not shown). Intramolecular crosslinked products of both the 49/53 kDa and 33 kDa proteins are also apparent in this figure.

4. CONCLUSIONS

The results presented in this communication clearly demonstrate that the extrinsic 33 kDa pro-

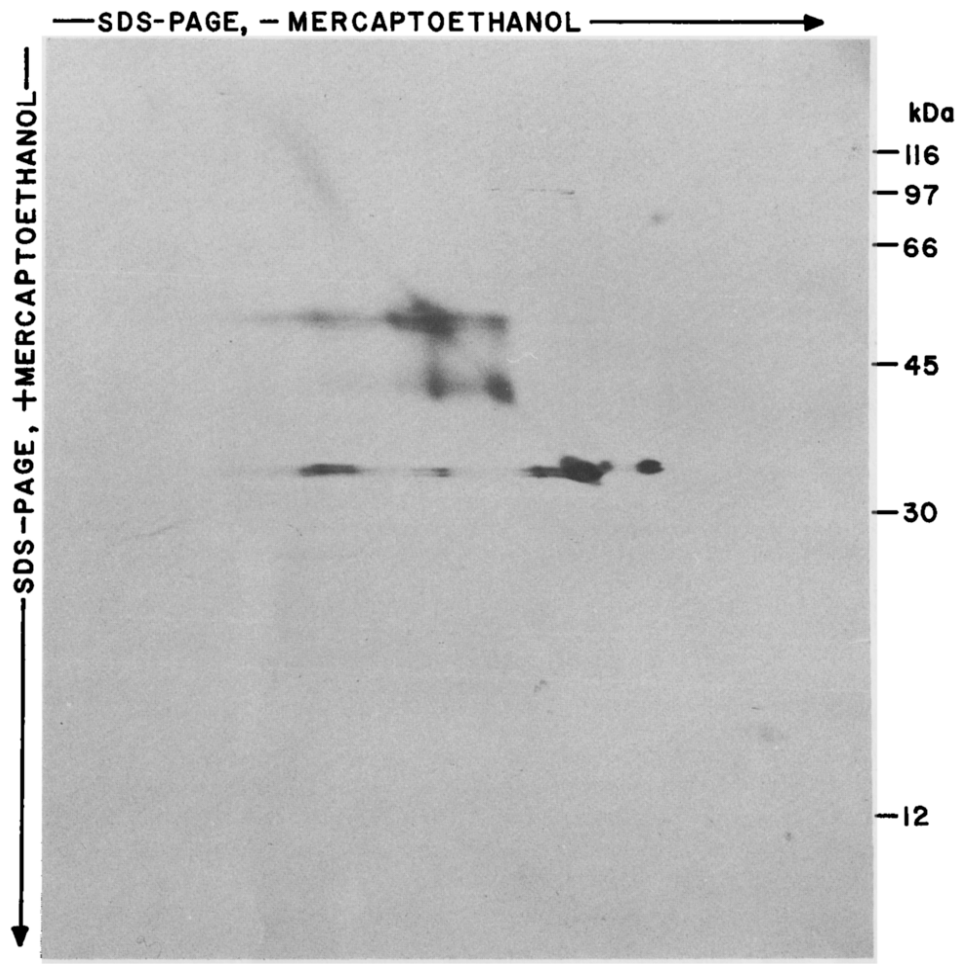

Fig.3. Two-dimensional diagonal electrophoresis of oxygen-evolving reaction center core complex treated with the crosslinker DTSP. After DTSP treatment (500 μM for 100 min), constituent polypeptides were separated by SDS-PAGE without mercaptoethanol (first dimension), followed by SDS-PAGE in the presence of mercaptoethanol (second dimension). After electrophoresis the separated proteins were transferred to nitrocellulose paper which was blocked and probed with the antibodies FAC2 and α33, simultaneously. Proteins which reacted with these antibodies were then visualized as described previously. Protein spots which represent the 49/53 kDa apoprotein of CPa1 and the extrinsic 33 kDa component are found as a vertical series below the diagonal, indicating intermolecular crosslinks between these two components. The crosslinked complex migrates with an apparent molecular mass of 101 kDa in the first dimension. The location of molecular mass standards are shown to the right.

tein and the apoprotein of CPa1 are closely associated in PS II. The NHS-biotinylation experiment demonstrates that there exist amino groups on the CPa1 apoprotein which become exposed upon removal of the 33 kDa extrinsic protein. The EDC crosslinking experiments strongly suggest that regions of complementary charge exist on these two components which allow crosslinking by this reagent. The DTSP experiments demonstrate that the extrinsic 33 kDa and the CPa1 apoprotein can be reversibly crosslinked. Morris and Herrmann [20] have speculated that the large extrinsic loop of the apoprotein of CPa1 may interact with the extrinsic polypeptides associated with the oxygen-evolving complex. The results that we present here, as well as our earlier work using trypsin and the monoclonal antibody FAC2 as probes of this association, support this speculation. We suggest that the 49/53 kDa apoprotein of CPa1, in addition to its role as a light-harvesting antenna, may serve as a binding protein for the extrinsic 33 kDa polypeptide. Other possible roles for the CPa1 apoprotein, such as manganese binding, cannot be excluded at this time.

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