Wang, T., & King, T. E. (1982) *Biochem. Biophys. Res. Commun. 104*, 591–596.
Wikstrom, M., Krab, K., & Saraste, M. (1981) *Annu. Rev. Biochem. 50*, 623–655.
Yu, L., & Yu, C. A. (1982) *J. Biol. Chem. 257*, 10215–10221.
Yu, L, Yang, F. D., & Yu, C. A. (1985) *J. Biol. Chem. 260*, 963–973.
Zhu, Q. S., Berden, J. A., DeVries, S., Folkers, K., Porter, T., & Slater, E. C. (1982) *Biochim. Biophys. Acta 682*, 160–167.

Isolation of a Manganese-Containing Protein Complex from Photosystem II Preparations of Spinach†

Neil R. Bowlby and Wayne D. Frasch*
Department of Cellular and Molecular Biology, Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109
Received April 11, 1985

ABSTRACT: Purified ^{125}I-labeled 33-kDa protein binds to calcium-washed photosystem II preparations at high-affinity and low-affinity binding sites. Filling 70% of the high-affinity site with 33-kDa protein induces 63% of the maximum achievable reconstitution of O_2-evolving activity. When *N*-succinimidyl [(4-azidophenyl)dithio]propionate modified 33-kDa protein was reconstituted into Ca(II)-washed membranes under conditions that primarily filled the high-affinity site and then cross-linked to adjacent proteins by illumination of the photoaffinity label, a cross-linked protein complex was formed that could be solubilized from the membranes with sodium dodecyl sulfate. The protein complex consisted of 22-, 24-, 26-, 28-, 29-, and 31-kDa proteins cross-linked to the 33-kDa protein and contained about 3–4 mol of Mn/mol of protein.

The light reactions of photosystem II create strong oxidants that are ultimately reduced by the oxidation of water to molecular oxygen, a reaction catalyzed by the oxygen-evolving complex (OEC).[1] The OEC contains four manganese (Cheniae & Martin, 1970), which can be released by any of several treatments that inactivate O_2-evolving activity. These treatments include, among others, incubation of the membranes at high pH (Briantais et al., 1977) or in Tris (Yamashita & Butler, 1968). However, cross-linking the proteins in the thylakoid membranes with low concentrations of glutaraldehyde reduces the extent of flash-induced inactivation of oxygen-evolving activity by Tris to one-fourth of the extent in un-cross-linked membranes (Frasch & Cheniae, 1980). This resistance to inactivation apparently results from an increased stabilization of the manganese that is associated with the proteins of the OEC caused by the cross-linked proteins.

The polypeptide composition of this manganoenzyme has not been elucidated though progress has been made in recent years. Purified membrane particles (Bertold et al., 1981; Kuwabara & Murata, 1982) and PS II core complexes (Tang & Satoh, 1985; Ikeuchi et al., 1985), which catalyze the electron transport reactions of PS II, have led to the characterization of many of the proteins that mediate these reactions. In the former preparations, light-harvesting complex proteins (25–29 kDa) are the proteins found in greatest abundance (Arntzen, 1978) but are absent in the core complexes. Two proteins run as diffusely staining bands in SDS–PAGE. First is the herbicide-binding protein called D_1 (32 kDa), which has been identified to contain the binding site for the second quinone acceptor, Q_B (Pfister et al., 1981). Second is the D_2 protein (34 kDa), which has been hypothesized to be on the oxidizing side of PS II. Cytochrome *b*-559, associated with PS II, has been purified as a 9-kDa protein (Metz et al., 1983; Widger et al., 1984). The chlorophyll proteins CPa-1 (47 kDa) and CPa-2 (43 kDa) have been suggested to be the reaction center and subantenna for PS II, respectively (Delepelaire & Chua, 1979; Green & Cam, 1983; Nakatani et al., 1984), although recent evidence suggests a close correlation between the reaction center proteins of *Rhodopseudomonas viridis* with D_1 and D_2 of PS II (R. Sayre, personal communication; A. Trebs, personal communication).

Three extrinsic membrane proteins (17, 23, and 33 kDa) are found on the inside of the thylakoid membrane and serve functional roles on the oxidizing side of PS II. Akerlund et al. (1982) found that when the 17- and 23-kDa proteins were removed from inside-out thylakoid vesicles by washing in dilute salt, the ability to evolve oxygen was lost. Oxygen-evolving activity was partially restored when the 23-kDa protein was reconstituted with the membranes (Akerlund et al., 1982). Yamamoto et al. (1981) observed that Tris causes the release of the three extrinsic proteins as well as the manganese from the membrane, which results in an irreversible loss of O_2-evolving activity. However, Ono and Inoue (1983) recently found that washing the membranes in high concentrations of calcium removes the three extrinsic proteins from PS II preparations without substantial loss of manganese. A significant amount of O_2-evolving capacity can be restored to Ca-washed membranes by reconstitution with the 33-kDa protein, and activity can be enhanced further by the addition of the 23-kDa protein or Ca(II) (Kuwabara et al., 1985).

Because the 23-kDa protein (Yamamoto et al., 1983; Kuwabara & Murata, 1983) as well as the 33-kDa protein can

† This work was supported by grants from the Office of Energy Research and a National Institutes of Health Biomedical Research Support Grant to the Vice President for Research to W.D.F. and by a Newcombe fellowship to N.R.B.

[1] Abbreviations: PS II, photosystem II; PAGE, polyacrylamide gel electrophoresis; OEC, oxygen-evolving complex; SDS, sodium dodecyl sulfate; SADP, *N*-succinimidyl [(4-azidophenyl)dithio]propionate; SMN, 400 mM sucrose, 50 mM MES, 5 mM $MgCl_2$, and 10 mM NaCl, pH 6.0; MES, 4-morpholineethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/86/0425-1402$01.50/0

be reversibly extracted from PS II preparations without significant loss of manganese, it is believed that these proteins bind to a hydrophobic core of proteins that contain the metal cluster and are directly responsible for oxygen evolution; the 23- and 33-kDa proteins probably serve, then, to stabilize this enzyme complex. The 23-kDa protein is believed to be distal to the 33-kDa protein because incubation of the membranes in 1 M NaCl at pH 6.5 removes the 17- and 23-kDa proteins but not the 33-kDa protein (Kuwabara & Murata, 1983) and because, when extracted under oxidizing conditions, the 33-kDa protein retains two manganese (Abramowicz & Dismukes, 1984). It has been suggested that a 34-kDa protein provides part of the manganese binding site from studies with corn mutants, which correlate the absence of this protein with the absence of manganese and O_2-evolving activity (Bricker et al., 1983). To identify the proteins that exist in close proximity to the 23- and 33-kDa proteins, Ljundberg et al. (1984) made antibodies to these extrinsic proteins and then analyzed the proteins that coprecipitated with the antibodies following partial solubilization of the membranes with detergent. The experiments demonstrated a close association of 24-, and 22-, and 10-kDa proteins with the two extrinsic proteins. However, none of the precipitated complexes contained more than trace amounts of manganese.

In the experiments described here, we have examined the constituents that compose the binding site for the 33-kDa protein during reconstitution of O_2-evolving activity in calcium-washed PS II preparations. A high-affinity binding site was observed that correlated with the reconstitution of enzymatic activity. This site was probed with 33-kDa protein modified to contain covalent adducts of the heterobifunctional cross-linking reagent *N*-succinimidyl [(4-azidophenyl)dithio]propionate (SADP). The 33-kDa protein modified to contain approximately eight adducts of SADP was able to reconstitute O_2-evolving activity. A single cross-linked protein complex was formed following reconstitution and cross-linking by illumination. This complex, which could be solubilized from the membrane with SDS, was found to contain about three to four manganese atoms and was composed of 22-, 24-, 26-, 28-, 29-, 31-, and 33-kDa proteins.

Experimental Procedures

Membrane preparations enriched in photosystem II were isolated from spinach by a modification of the Bertold et al. (1981) procedure employing a wash buffer of pH 6.5 and only one extraction in Triton X-100. The membranes were stored at −70 °C in 400 mM sucrose, 50 mM MES, 5 mM $MgCl_2$, and 10 mM NaCl, pH 6.0 (SMN). These PS II preparations commonly exhibited rates of O_2 evolution of about 450–640 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Rates of O_2 evolution were measured in a Clark-type electrode with 20 μg of Chl/mL in a 1-mL reaction vessel, 250 μM dichlorobenzoquinone and 3 mM ferricyanide as an electron acceptor, and 400 mM sucrose, 50 mM MES, pH 6.0, and 10 mM NaCl.

Purification of the 33-kDa Protein. The purified PS II preparations were diluted to 100 μg of Chl/mL in SMN buffer containing 1 M NaCl for 1 h in darkness at 4 °C to extract the 17- and 23-kDa proteins as described by Kuwabara and Murata (1982). The suspension was centrifuged at 20000*g* for 10 min to pellet the membranes, which were resuspended in the extraction buffer described by Ono and Inoue (1983) for 1 h in darkness at 4 °C to solubilize the 33-kDa protein from the membranes. The membranes were removed by centrifugation at 40000*g* for 30 min, and the supernatant that contained the 33-kDa protein was concentrated by pressure dialysis with an Amicon YM5 membrane. The $CaCl_2$ was removed from the concentrated sample by column chromatography on Sephadex G-25 (0.8 × 50 cm) equilibrated in 50 mM MES, pH 6.0, and 50 mM NaCl. Further purification was achieved by chromatography on DEAE-Sephadex A-50 (0.8 × 15 cm) in 50 mM MES, pH 6.0, and with a linear gradient of 50–300 mM NaCl. A single peak eluted from the column at 150–175 mM NaCl, which was shown by SDS–PAGE to be the 33-kDa protein. The protein was further purified to 98% purity by high-performance liquid chromatography on a PSM-150 Diol column (Du Pont) and then stored at 4 °C with 1 mM PMSF.

Protein Cross-Linking. The following steps were carried out in darkness. The purified, desalted 33-kDa protein was added slowly to 10 mM SADP (Pierce Chemicals) in dimethyl sulfoxide such that the final solution was >70% $(CH_3)_2SO$. After reaction for 30 min at room temperature, the $(CH_3)_2SO$ and unreacted SADP were removed by chromatography on Sephadex G-25 (0.8 × 15 cm) equilibrated in 40 mM MES, pH 6.5. The resulting SADP-modified protein (10 μg) was added to the PS II preparation, which had preincubated in the extraction buffer at 4 °C for 1 h, pelleted by centrifugation, and resuspended in SMN with 15 mM $CaCl_2$. The SADP-modified protein was allowed to bind to the calcium-washed membranes at final concentrations of 1.25 μg of protein/mL and 0.25 mg of Chl/mL, respectively, at 4 °C for 1 h. The PS II preparation was then pelleted by centrifugation and resuspended in SMN to a concentration of 0.1 mg Chl/mL just prior to illumination with 254-nm light for 5 min at 4 °C to cross-link the proteins. The cross-linked membranes were pelleted by centrifugation and resuspended in 40 mM MES, pH 6.5, containing 1% SDS (w/v) to a final concentration of 2 mg of Chl/mL and incubated for 90 min at 37 °C to solubilize the proteins. Membrane fragments remaining after solubilization were removed by centrifugation at 30000*g* for 15 min, and the supernatant was applied to a Sephadex G-100 column (1.5 × 70 cm) equilibrated with the MES–SDS buffer. The fractions containing the cross-linked complex were stored at 4 °C.

Analysis of Cross-Linked Proteins by Electrophoresis. The separation of proteins by SDS–PAGE was done following the procedure of Chua (1980) with 15 V/cm. The constituent subunits of the cross-linked complex were examined by obtaining a final purification of the cross-linked complex by electrophoresis in 10% polyacrylamide. The lane containing the protein was removed from the gel in the first dimension and soaked for 2 min in 20 mM 2-mercaptoethanol before being mounted horizontally onto a slab gel that was composed of a 12% stacking gel and a 15% resolving gel. The subunits were separated in the second dimension with an upper reservoir buffer that contained 20 mM 2-mercaptoethanol to ensure complete reduction of the dithiol bonds of SADP. The gels were stained with silver nitrate as described (Sammons et al., 1981).

Protein concentrations were determined by the Bradford (1976) assay for samples that did not contain SDS and by ultraviolet absorption for samples with SDS by using the equation

$$\text{mg of protein/mL} = 1.5A_{280} - 0.75A_{260}$$

as modified from Layne (1957). Quantitation of manganese was done by neutron activation at the Phoenix Memorial Reactor by irradiating the samples by a source of neutrons for 4 min and then counting the γ emission from the sample at 846 KEV (the energy of the Compton edge for the γ emitted from ^{56}Mn). The 33-kDa protein was labeled with ^{125}I by iodogen (Pierce Chemicals) and separated from excess un-

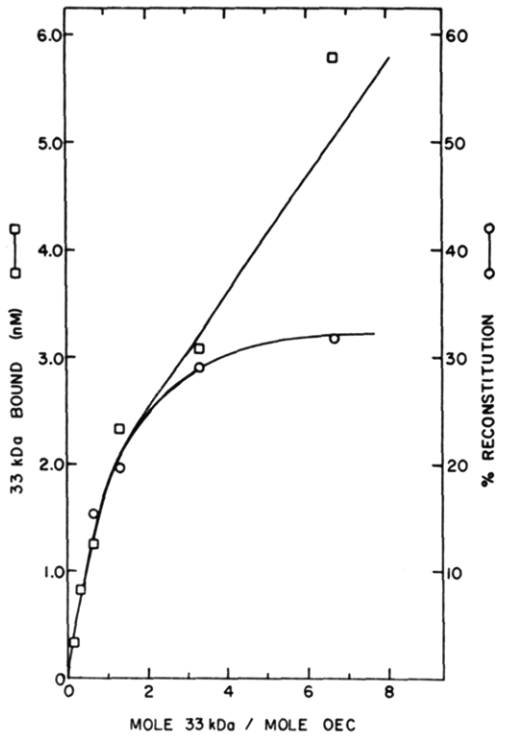

FIGURE 1: Dependence of reconstitution of O_2-evolving activity (circles) and binding of ^{125}I-labeled 33-kDa protein (squares) on the amount of 33-kDa protein added to $CaCl_2$-washed PS II preparations. The membranes (250 μg of Chl/mL) were incubated with the protein for 1 h at 4 °C in darkness and then washed free of unbound protein and counted or assayed for O_2-evolving activity.

reacted reagent by chromatography with Bio-Gel P-30.

RESULTS

The extent of binding of the 33-kDa protein to calcium-washed PS II preparations was examined by using protein that had been radioactively labeled with ^{125}I. As shown in Figure 1, the amount of protein bound as a function of protein added increased in a biphasic manner. At low concentrations, the protein bound with high affinity. However, when the 33-kDa protein was added to the membranes at ratios greater than 3 mol of 33-kDa protein/mol of OEC [assuming 230 Chl/OEC as shown by Lam et al. (1983)], the protein bound with lower affinity.

The extent of binding of the 33-kDa protein was related to the extent of reconstitution of O_2-evolving activity in the calcium-washed PS II preparations as shown by the open circles in Figure 1. Half-maximal reconstitution was achieved with a ratio of 1 mol of protein added per mole of OEC. Addition of the 33-kDa protein to calcium-washed membranes such that 70% of the high-affinity sites were filled caused the recovery of 63% of the maximum achievable reconstitution of O_2-evolving activity. This suggests that the binding of the 33-kDa protein to this site is responsible for reconstitution of the enzymatic activity.

To establish the polypeptide composition of the binding site responsible for the reconstitution mediated by the 33-kDa protein, the purified 33-kDa protein was covalently modified in the dark to contain adducts of SADP. Under these conditions, the succinimidyl group of SADP reacts with the amine of a lysine to form a covalent adduct that contains an azido group capable of acting as a photoaffinity label. From the extinction coefficient of SADP (ϵ'_{266} = 14 300), the number of modified lysines on the 33-kDa protein could be determined. As shown in Figure 2, the number of lysines modified was controlled by incubating the protein in various concentrations

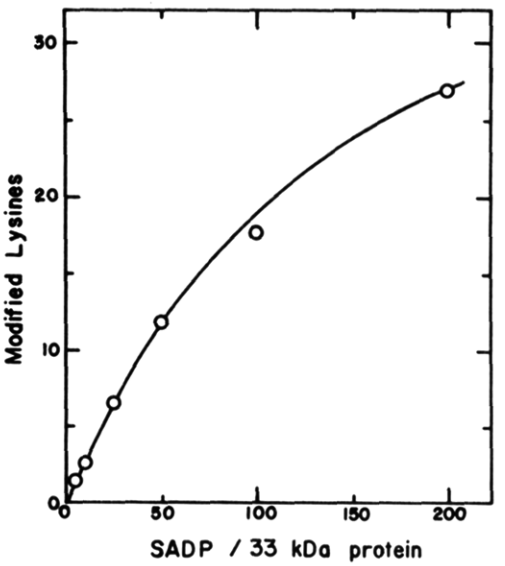

FIGURE 2: Dependence of the number of lysines modified by SADP on the mole ratio of SADP added. The protein samples each containing 66 μg of 33-kDa protein were incubated in the mole ratios of SADP indicated for 30 min at 20 °C in the dark, and the reaction was quenched with a 10 M excess of methylamine. Unreacted SADP was removed by centrifugation chromatography with Sephadex G-25 equilibrated in 40 mM MES, pH 6.5, prior to determination of absorbance at 266 nm.

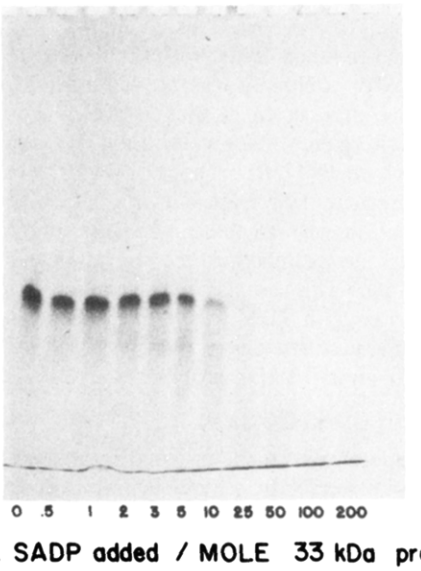

FIGURE 3: Native gel electrophoresis of the 33-kDa protein modified with various amounts of SADP. The protein (6 μg) that was modified by SADP in Figure 2 was loaded onto a 5% polyacrylamide gel in 0.375 M Tris-HCl buffer, pH 8.9, and separated by electrophoresis at 200 V for 3 h. Upper and lower reservoir buffers contained 40 mM glycine and 20 mM Tris-HCl, pH 8.31. The proteins were stained with Coomassie Brilliant Blue G-250.

of the reagent. Twenty-eight of the thirty lysines on the protein were modified at a 200 molar excess of SADP over the 33-kDa protein.

The homogeneity of the modified 33-kDa protein was examined by native polyacrylamide electrophoresis (Figure 3). Native gels separate proteins primarily by charge, and because the modification of a lysine by SADP changes the charge of the side chain, the observed electrophoretic mobility is indicative of the number of lysine residues modified by the reagent. When 28 of the 30 lysines on the protein were modified, a sharp band of protein was observed that had R_f 1.0 whereas the R_f of the unmodified protein was 0.56. Figure 3 also shows that when a limited number of the lysines are modified, some

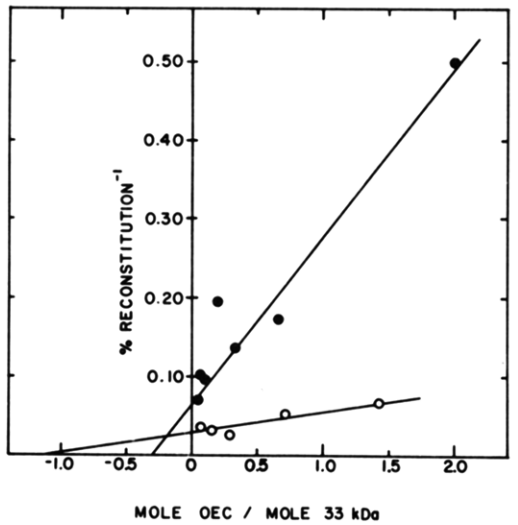

FIGURE 4: Double-reciprocal plot of the dependence of the extent of reconstitution of O_2 evolution on the concentration of 33-kDa protein added to $CaCl_2$-washed PS II preparations: (open circles) unmodified 33-kDa protein; (closed circles) 33-kDa protein containing about eight adducts of SADP.

of these side chains are more accessible to the reagent than others. At ratios of 0.5–2 mol of SADP added per mole of protein, a single lysine becomes modified. At ratios of 3–25 SADP/protein, about four more lysines react. The protein having about five covalent adducts runs as a somewhat diffuse band, suggesting that there is some variability as to which lysines in the protein sequence are modified. It is likely that about four of the five modified lysines have the same position in the sequence of the protein. At higher concentrations of SADP to protein, there is another group of lysines that become accessible to the reagent before the protein reacts exhaustively with the SADP.

The effect of modification of the protein on the ability to reconstitute O_2-evolving activity is shown in Figure 4. The ability to reconstitute O_2 evolution as a function of the amount of protein added is expressed as a double-reciprocal plot. In this experiment, the solid circles indicate reconstitution with 33-kDa protein, which had approximately eight adducts of SADP with negligible amounts of unmodified protein present. The protein modified in this manner was able to reconstitute enzymatic activity although it is evident from the slope and intercept that the affinity of the modified protein for the binding site and the functional capacity of this protein have been somewhat altered. When 15 or more of the lysines were modified with SADP, the protein was no longer capable of reconstituting O_2-evolving activity (data not shown).

Following the reconstitution of calcium-washed PS II preparations with the SADP-labeled 33-kDa protein under conditions in which the native protein binds specifically to the high-affinity site, illumination of the sample with UV light cross-linked the bound 33-kDa protein to other membrane proteins that were within 8 Å. The cross-linked proteins were partially purified from the membrane by solubilization in SDS and chromatography with Sephadex G-100. Micelles that contained detergent and protein eluted following the void volume. As shown in Figure 5 (lane 3, band c), the micelles were unable to enter a 10% SDS–PAGE gel. Subsequent fractions from the G-100 column were enriched in a 75-kDa band (lane 3, band d) that was absent in SDS–PAGE gels of membranes that had not been cross-linked.

The strip of gel adjacent to that shown in lane 3 of Figure 5 was not stained but was subjected to electrophoresis in the second dimension following saturation with 2-mercaptoethanol

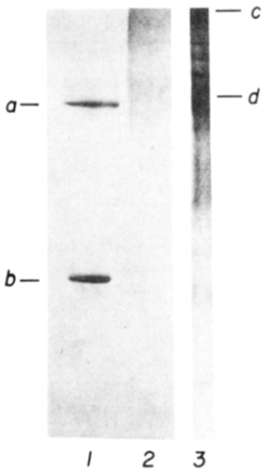

FIGURE 5: Cross-linked protein complex after solubilization from PS II membranes by SDS. The proteins were separated by SDS–PAGE on a 10% gel. (Lane 3) Fraction enriched in the cross-linked complex (band d) that eluted from a Sephadex G-100 column (see Experimental Procedures). This fraction also contains protein–detergent micelles (band c). (Lane 2) Fraction enriched in the cross-linked complex obtained by chromatography of the protein from lane 3 with Sepharose CL-4B (200 mesh). The proteins were separated by SDS–PAGE as for lane 3. (Lane 1) Molecular weight standards: (a) bovine serum albumin (66 kDa); (b) α-chymotrypsin (25 kDa).

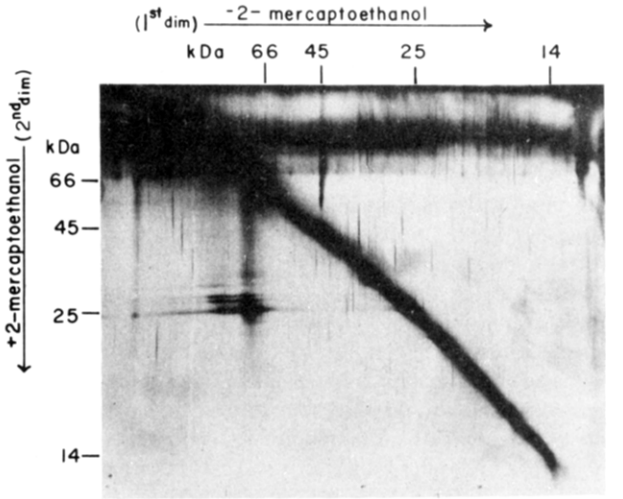

FIGURE 6: Protein constituents of the 75-kDa cross-linked complex. The constituents of the complex were separated by SDS–PAGE with 15% polyacrylamide and 20 mM 2-mercaptoethanol (for subunit dissociation) in the second (vertical) dimension after separation by electrophoresis in 10% polyacrylamide in the first (horizontal) dimension. Molecular weights shown were determined by coelectrophoresis of the following standards: bovine serum albumin (66 kDa), egg albumin (45 kDa), α-chymotrypsin (25 kDa), and lysozyme (14 kDa).

to reduce the dithiol linkages of SADP. With this two-dimensional gel (Figure 6), it was possible to resolve the protein constituents of the purified complex. Because un-cross-linked proteins have the same relative mobilities in each dimension of the gel, they appear on a diagonal. However, cross-linked proteins migrate as a higher molecular weight species in the first dimension but separate into the individual components in the second dimension, and thus, these constituents appear below the diagonal. The 33-kDa protein was found to cross-link to 22-, 24-, 26-, 28-, 29-, and 31-kDa proteins. Control experiments using whole PS II particles or Ca(II)-washed particles showed no evidence of native complexes labile to 2-mercaptoethanol cleavage (not shown).

The cross-linked complex was further purified by chromatography with Sepharose CL-4B (200 mesh) in the presence

Table I: Quantitation of Mn As Determined by Neutron Activation

sample	nmol of Mn	mol of Mn/230 Chl	mol of Mn/mol of complex
complex	3.7 ± 0.70		3–4[a]
buffer	0.5 ± 0.40		
PS II membranes	4.9 ± 0.39	4.3	

[a] Calculated with protein concentrations as determined under Experimental Procedures with 16% purity and an effective M_r of 75 000 for the complex.

of SDS to a purity of about 16% as determined by densitometry of the SDS–PAGE gel shown in Figure 5 (lane 2). The sample contained only two major contaminants that were approximately 36 and 25 kDa. Purification of the protein by the two chromatography columns also served to remove Mn(II) free in solution, which may have been released by the detergent. The manganese content of the cross-linked complex, as determined by neutron activation, was found to be 3.7 nmol of Mn or 6.49 nmol of Mn/mg of protein as shown in Table I. The concentration of the metal was about 7-fold higher in the protein sample than in the buffer used to equilibrate the columns. This Mn was found to be associated specifically with the cross-linked complex as determined by Mn analysis of the cross-linked band from the first-dimension gel (Figure 5, lane 3). The complex was found to contain about 3–4 mol of manganese/mol of protein when corrected for purity (see Discussion).

Discussion

The results presented here suggest that at least some of the proteins that compose the cross-linked complex are intimately associated with the oxygen-evolving system: (i) the correlation between the extent of binding of ^{125}I-labeled 33-kDa protein to the high-affinity site and the extent of reconstitution of O_2-evolving activity suggests that binding to the high-affinity site is responsible for O_2-evolving activity (Figure 1); (ii) the purified cross-linked complex contains three to four manganese atoms (Table I); (iii) the conditions for forming the complex were chosen to maximize the ratio of functional vs. nonfunctional rebinding of the modified protein to the salt-washed membranes (Figures 1–4); (iv) cross-linking under these conditions yields what appears, by our method of purification, to be a single species.

Although conditions were chosen to minimize the nonfunctional rebinding of protein and only one major cross-linked species was apparent, the extent of nonfunctional binding in these experiments was significant. The cross-linked complex described here contains significant amounts of manganese, which suggests that these proteins contain the site of functional binding. If this complex results from functional rebinding, there are two possible ways in which the nonspecific rebinding of SADP-modified 33-kDa protein is visible on the 2-D gels. First, the nonspecific rebinding occurs to such a diverse population of sites that no single site is found in high abundance. This would cause the nonspecific binding to be responsible for the diffusely stained background below the diagonal in Figure 6. Second, the nonspecific binding forms a complex that has the same mobility as the manganese-containing complex in the first dimension of the SDS–PAGE. Our preliminary evidence suggest that both types of nonspecific complexes are present, and work is currently under way to characterize the latter nonspecific complex. We also cannot exclude the possibility that, while bound to the site of reconstitution, a small fraction of the modified 33-kDa protein cross-linked primarily to proteins not involved with water oxidation to form cross-linked complexes with a similar molecular weight to that of the OEC-specific cross-linked complex. However, the abundance of these other complexes must be small in light of the calculated ratio of manganese to protein.

The complex is composed of 22-, 24-, 26-, 28-, 29-, 31-, and the 33-kDa proteins. The identities of these proteins are presently not known, and it is probable that not all of the constituents of the complex participate in O_2 evolution. The 26-, 28-, and 29-kDa proteins are probably LHC isoproteins, which must be in close association with the reaction center for efficient transfer of energy from antenna to P680. However, the possibility exists that these proteins have a specific function in the OEC and have not been observed previously in one-dimensional SDS–PAGE due to the abundance of LHCP. The 22- and 24-kDa proteins have been implicated to be associated with the 33-kDa protein by a previous study involving coimmunoprecipitation (Ljundberg et al., 1984), although these precipitates did not contain significant amounts of manganese. A 22-kDa protein has also been found to be a component of highly purified PS II core complexes, which retain the ability to oxidize water (Tang & Satoh, 1985).

Ikeuchi et al. (1985) have recently isolated a PS II core complex that lacks the 22–29-kDa proteins yet retains manganese and the ability to evolve oxygen. These workers concluded that the OEC is not distinct from the reaction center. The absence of the 47- and 43-kDa proteins from the cross-linked complex presented here indicates that the binding site of the 33-kDa proteins is greater than 8 Å from either of these proteins. In light of these results, it is possible that the manganese in the cross-linked protein complex is associated with only the 33- and 31-kDa proteins. The 31-kDa protein runs as a well-defined band, which suggests that it is not D_1, the herbicide-binding protein.

Several lines of evidence support the hypothesis that the manganese associated with the OEC has been stabilized in the preparation reported here as a result of the formation of covalent bridges between the proteins surrounding the metal cluster including: (i) the metal remains associated with the protein following purification by two successive chromatography columns or SDS–PAGE in the absence of thiol-reducing agent, (ii) the manganese is associated directly with the cross-linked complex as determined by analysis of the cross-linked band in the polyacrylamide electrophoresis gel, and (iii) the cross-linked complex was formed under conditions that maximized functional rebinding.

Ono and Inoue (1983) found that the amount of manganese released from the membranes by the Ca(II) wash was negligible. Thus, it is unlikely that manganese dissociated from the OEC and reassociated with a nonspecific site prior to cross-linking. Very low amounts of SADP-modified 33-kDa protein were used to rebind to the membranes such that only a small fraction of the available functional sites were filled. Consequently, a significant amount of manganese was released during detergent fractionation of the membranes. It is possible that some of this manganese may have reassociated with the cross-linked protein complex following solubilization. Any manganese bound adventitiously to this complex would be required to be very tightly bound to survive the purification by chromatography and electrophoresis. To date, the only protein in PSII preparations to copurify with manganese has been the 33-kDa protein (Abramowicz & Dismukes, 1984), which has an affinity for the metal only under oxidizing conditions. Such oxidizing conditions were not employed in the purification of the cross-linked complex described here. Thus, we conclude that the manganese associated with the

complex represents either functional manganese or manganese bound to a previously unreported tight binding site.

The cross-linked protein complex has an apparent M_r of 75 000 as determined by SDS–PAGE (Figure 5). However, the sum of the protein constituents of the complex (Figure 6) suggests that SDS is not capable of complete denaturation of these proteins when they are cross-linked such that the true molecular weight of the complex is somewhat higher than 75 000. The accuracy of the quantitation of the mole ratio of manganese to protein is limited not only by the uncertainty of the molecular weight but also by the error inherent in the methods currently available to determine the protein concentration of the complex. We have used the absorbance at 280 nm to determine protein concentration because this method does not exhibit interference from the detergent and has been shown to vary less from protein to protein. To estimate the mole ratio of manganese to protein, we have used the apparent M_r of 75 000, which was obtained from the mobility of the complex on SDS–PAGE. The true molecular weight of the complex is somewhat greater as determined by the analysis of the subunits. If a 1:1 stoichiometry is assumed for each protein in the complex, the aggregate molecular weight is about 200 000. However, it is unlikely that such a large complex would have the mobility with SDS–PAGE that is observed and suggests that the relative abundance of the individual subunits is somewhat variable and/or that some other protein complexes have comigrated in the first dimension of the gel. This heterogeneity is apparent in Figure 6 by the variability in the size and intensity of the bands that lie below the diagonal. The greatest variation is observed in the proteins in the molecular weight range of the LHC isoproteins, which suggests that each complex may only contain one of these very similar proteins. To obtain a mole ratio of four Mn/complex, the molecular weight of the complex would be about 100 000.

ACKNOWLEDGMENTS

We thank John Jones, Director of the Phoenix Memorial Laboratory at The University of Michigan, for his assistance with neutron activation analysis.

REFERENCES

Abramowicz, D. A., & Dismukes, G. C. (1984) *Biochim. Biophys. Acta 765*, 318–328.

Åkerlund, H.-E., Jansson, C., & Andersson, B. (1982) *Biochim. Biophys. Acta 681*, 1–10.

Arntzen, C. J. (1978) *Curr. Top. Bioenerg. 8*, 111–160.

Bertold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.

Bradford, M. A. (1976) *Anal. Biochem. 72*, 248–254.

Briantais, J.-M., Vernotte, C., Lavergne, J., & Arntzen, C. J. (1977) *Biochim. Biophys. Acta 461*, 61–74.

Bricker, T. M., Metz, T. G., & Sherman, L. A. (1983) *Biochim. Biophys. Acta 724*, 447–455.

Cheniae, G. M., & Martin, I. F. (1970) *Biochim. Biophys. Acta 197*, 219–239.

Chua, N.-H. (1980) *Methods Enzymol. 69*, 434–446.

Delepelaire, P., & Chua, N.-H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 111–115.

Frasch, W. D., & Cheniae, G. M. (1980) *Plant Physiol. 65*, 735–745.

Green, B., & Camm, E. L. (1983) *Abstract, Sixth International Congress on Photosynthesis*, pp 101–108, Martinus Nijhoff/Dr. W. Junk, The Hague, The Netherlands.

Ikeuchi, M., Yuasa, M., & Inoue, Y. (1985) *FEBS Lett. 185*, 316–322.

Kuwabara, T., & Murata, N. (1982) *Plant Cell Physiol. 23*, 533–539.

Kuwabara, T., & Murata, N. (1983) *Plant Cell Physiol. 24*, 741–747.

Kuwabara, T., Miyao, M., Murata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 806*, 283–289.

Lam, E., Baltimore, B., Ortiz, W., Chollar, S., Melis, A., & Malkin, R. (1983) *Biochim. Biophys. Acta 724*, 201–211.

Layne, E. (1957) *Methods Enzymol. 3*, 447–454.

Ljundberg, U., Åkerlund, H.-E., Larson, C., & Andersson, B. (1984) *Biochim. Biophys. Acta 767*, 145–152.

Menke, W., & Koenig, F. (1980) *Methods Enzymol. 69*, 446–452.

Metz, J. G., Ulmer, G., Bricker, T. M., & Miles, D. (1983) *Biochim. Biophys. Acta 726*, 203–209.

Nakatani, H. Y., Ke, B., Dolan, E., & Arntzen, C. J. (1984) *Biochim. Biophys. Acta 765*, 347–352.

Ono, T.-A., & Inoue, Y. (1983) *FEBS Lett. 164*, 255–260.

Pfister, K., Steinback, K. E., Gardner, G., & Arntzen, C. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 981–985.

Sammons, W., Adams, L. D., & Nishizawa, E. E. (1981) *Electrophoresis 2*, 135.

Tang, X-S., & Satoh, K. (1985) *FEBS Lett. 179*, 60–64.

Widger, W. R., Cramer, W. A., Hermodson, M., Gullifor, M., & Meyer, D. (1984) in *Advances in Photosynthesis Research* (Sybesma, C., Ed.) Vol. 1, pp 501–504, M. Nijhoff/Dr. W. Junk, The Hague.

Yamamoto, Y., Doi, M., Tamura, N., & Nishimura, M. (1981) *FEBS Lett. 133*, 265–268.

Yamamoto, Y., Shimada, S., & Nishimura, M. (1983) *FEBS Lett. 151*, 49–53.

Yamashita, T., & Butler, W. L. (1968) *Plant Physiol. 43*, 1978–1986.