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Manganese-Binding Proteins of the Oxygen-Evolving Complex†

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ABSTRACT: The extrinsic 33-kDa protein (P_{33}) was cross-linked covalently to the binding site on P_{33}-depleted PSII preparations which is responsible for reconstitution of photosynthetic water oxidation after PSII preparations have been washed with 1 M $CaCl_2$. Conditions were found in which more than half of the cross-linked protein complexes formed in the PSII preparations retained the ability to catalyze the oxidation of water. The complex is composed of the P_{33} cross-linked to the D_1 and D_2 proteins and a 34-kDa protein, which is present in lower abundance than the other three proteins. After solubilization of the membranes with SDS and purification by preparative SDS-PAGE, the complex retains bound manganese and can catalyze the conversion of H_2O_2 to O_2. Calcium and chloride increased the catalase activity of the purified cross-linked complex while lanthanum or hydroxylamine abolished the activity. By use of the specific activity of the H_2O_2-dependent reaction to follow the extent of purification of the cross-linked complex, the most highly purified complex was determined to contain 0.34 μg of manganese/180 μg of protein. The mole ratio of Mn/protein was calculated to range from 3.6 to 4.5 depending on the assumed stoichiometry of the protein subunits. The results presented here provide direct evidence that one or more of the three proteins that have cross-linked to the P_{33} are responsible for binding the manganese of the oxygen-evolving complex.

The oxygen-evolving complex (OEC)[1] catalyzes the oxidation of water to molecular oxygen in order to supply electrons to the photosystem II (PSII) reaction center. Oxidants generated by the photoreactions of PSII drive the sequential advancement of the five intermediate states (S_0-S_4) of the OEC (Kok et al., 1970; Forbush et al., 1971). Four bound manganese are

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[1] Abbreviations: Chl, chlorophyll; LHC, light-harvesting chlorophyll proteins; BCA, bicinchoninic acid; LHCII*, the 120-kDa aggregate of LHC proteins; D_1, the herbicide-binding protein; D_2, the diffusely staining 34-kDa protein; MES, 4-morpholineethanesulfonic acid; OEC, oxygen-evolving complex; PSII, photosystem II; P_{33}, the extrinsic 33-kDa protein of photosystem II; PAGE, polyacrylamide gel electrophoresis; SADP, succinimidyl [(4-azidophenyl)dithio]propionate; SDS, sodium dodecyl sulfate; SSADP, sulfosuccinimidyl [(4-azidophenyl)dithio]-propionate.

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central to the process of water oxidation (Cheniae & Martin, 1970) and give rise to low-temperature EPR signals centered at $g = 2.0$ (the multiline signal) (Dismukes & Siderer, 1981) and at $g = 4.1$ (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984) in the S_2 state. Photosynthetic O_2-evolving activity also requires calcium (Nakatani, 1984; Ghanotakis et al., 1984; Miyao & Murata, 1984) and chloride (Izawa et al., 1969).

Three extrinsic polypeptides with molecular masses of 17, 23, and 33 kDa (P_{17}, P_{23}, and P_{33}) were shown to contribute to the normal function of the OEC by the observation that the ability to evolve oxygen was lost upon removal of these proteins from the membrane (Åkerlund & Jansson, 1981). Many treatments that solubilize these proteins from the membrane release the functional manganese and cause the irreversible loss of enzymatic activity. However, washing the membranes with 1 M $CaCl_2$ (Ono & Inoue, 1983) or with 1 M NaCl (Åkerlund et al., 1982) will remove the P_{33} and/or the P_{23} and P_{17}, respectively, without loss of manganese. Although these washes inhibit the OEC, readdition of the P_{33} or P_{23} to $CaCl_2$-washed or NaCl-washed preparations, respectively, partially reconstitutes O_2-evolution activity (Ono & Inoue, 1984; Åkerlund et al., 1982).

The P_{23}-depleted membranes obtained by a NaCl wash retain the ability to advance to the S_3 state with a 2-fold increase in the α miss parameter and can catalyze the $S_3 \rightarrow S_4 \rightarrow S_0 + O_2$ transition at a rate that is decreased by about 5-fold (Dekker et al., 1984; Radmer et al., 1986). If elevated concentrations of Ca^{2+} and Cl^- are present during the assay, high rates of O_2 evolution can be obtained with this preparation in the absence of the P_{23} (Ghanotakis et al., 1984). Thus, the P_{23} is believed to increase the affinity of the OEC for Ca^{2+} and Cl^-.

Although the P_{23} and P_{17} can be removed without affecting the P_{33}, conditions that solubilize the P_{33} remove all three polypeptides, which suggests that the P_{33} is proximal to the PSII reaction center relative to the other proteins. The P_{33}-depleted membranes prepared by a $CaCl_2$ wash retain the ability to form the multiline signal (Styring et al., 1987; Miller et al., 1987) and can advance to the S_3 state with normal kinetics (Dekker et al., 1984; Miyao et al., 1987a), which suggests that other, intrinsic membrane proteins form the functional ligands to the manganese during these S-state transitions. Although the $S_3 \rightarrow S_4 \rightarrow S_0 + O_2$ step can occur when the P_{33} is absent, this transition is much slower, even at optimal Ca^{2+} and Cl^- concentrations (Miyao et al., 1987a). Since the P_{33} retains two bound manganese when solubilized under oxidizing conditions (Abramowicz & Dismukes, 1984), this protein may have a role in forming ligands to the manganese in the higher S states.

Reconstitution of water oxidation in $CaCl_2$-washed particles by the P_{33} results from the binding of this protein to a high-affinity binding site, which was measured directly by using ^{125}I-labeled P_{33} (Bowlby & Frasch, 1986). Consequently, low concentrations of the P_{33} bind selectively to the site of reconstitution. The proteins that compose the site of binding and reconstitution have been examined by cross-linking experiments using P_{33} which contained covalent adducts of the photoaffinity reagent SADP. Initially, the P_{33} was thought to have cross-linked to six polypeptides (Bowlby & Frasch, 1986). However, subsequent experiments (Bowlby & Frasch, 1987) showed that three of these polypeptides were not cross-linked but had copurified with the cross-linked complex as the aggregate of the light-harvesting chlorophyll proteins known as LHCII*. The cross-linked polypeptides were estimated to contain three to four manganese after purification from the membranes.

We now report conditions that optimize the O_2-evolving activity of PSII preparations during the formation of the cross-linked PSII protein complex such that cross-linked membranes are capable of catalyzing the oxidation of water. Under these conditions the P_{33} cross-linked to the D_1 and D_2 proteins as well as a 34-kDa protein. The most highly purified fraction of the cross-linked protein complex was determined to have a manganese/protein mole ratio of about 3.6 to 4.5 and retained the ability to convert H_2O_2 to O_2. These results suggest that the cross-linked complex is composed of the polypeptides that bind the manganese of the oxygen-evolving complex.

Experimental Procedures

The preparation of PSII particles and purification of the P_{33} were carried out as described (Bowlby & Frasch, 1986). PSII reaction center complex particles were prepared as described by Ghanotakis and Yocum (1986). Rates of photosynthetic or H_2O_2-dependent oxygen evolution were measured with a Clark-type electrode as described (Frasch & Mei, 1987). The protein content was determined by the bicinchoninic acid (BCA) assay described by Smith et al. (1985) with bovine serum albumin as a standard.

The abundance of manganese was determined by instrumental neutron activation analysis at the Ford Nuclear Reactor facilities of the Phoenix Memorial Laboratory, University of Michigan. Samples were placed in 1.5-mL polyethylene microfuge tubes and irradiated individually in the pneumatic irradiation system under automatic control of a Nuclear Data 6700 computer analyzer. Each sample was exposed to a neutron flux of 1.5×10^{13} n cm^{-2} s^{-1} for 3 min to generate ^{56}Mn, which has a half-life of 2.58 h. γ spectral analysis was performed with an Ortec Gamma-x intrinsic germanium detector arranged so that each sample was positioned exactly at the same distance from the detector. A 4K spectrum of γ irradiation from each sample was obtained by counting for 400 s. Counting began 6 min after the sample was removed from the neutron source, which allowed short-lived nuclides that might interfere with the analysis (primarily ^{25}Na, half-life 60 s) to decay. The γ radiation of ^{56}Mn at 0.846 MeV was used to determine the unknown quantities of manganese, which were compared to standards of $MnCl_2$.

The P_{33} was covalently modified to contain adducts of SSADP (Pierce Chemicals) by allowing 1 mg of P_{33} to react with 10 mM SSADP in 40 mM MES, pH 6.0, in a volume of 1 mL for 30 min at 25 °C. Excess unreacted SSADP was removed by the addition of 7 μmol of methylamine. The SSADP and methylamine were then removed by centrifugation chromatography with Sephadex G-25 in a 1-mL syringe as described (Penefsky, 1977). The extinction coefficient of SSADP ($\epsilon'_{266} = 14\,300$) was used to estimate the number of SSADP adducts formed per P_{33}.

To cross-link the proteins, PSII particles were incubated in 400 mM sucrose, 50 mM MES, pH 6.0, 5 mM $MgCl_2$, and 10 mM NaCl (SMN) which contained 1 M $CaCl_2$. The particles were pelleted by centrifugation such that the preparation was resuspended in SMN with SSADP-P_{33} at a ratio of 5 mg of Chl/mg of SSADP-P_{33} 10 min after the addition of 1 M $CaCl_2$. This ratio provided the maximal amount of reconstitution under conditions in which the extent of reconstitution of activity was directly proportional to the amount of P_{33} bound (Bowlby & Frasch, 1986). Cross-linking was initiated by illumination of the PSII preparations (0.1 mg of Chl/mL) that contained bound SSADP-P_{33} with 254-nm light

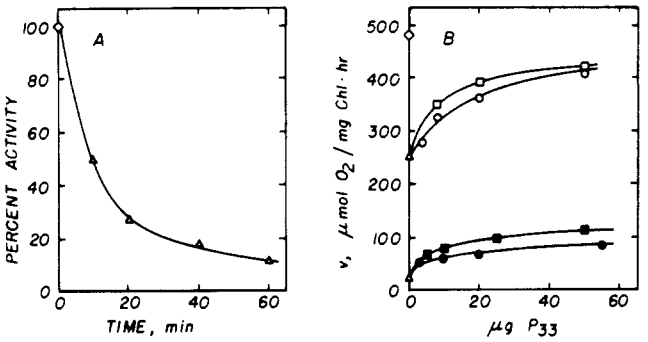

FIGURE 1: (A) Dependence of photosynthetic O_2-evolving activity on the duration of incubation of PSII preparations in 1 M $CaCl_2$. The membranes were suspended at a concentration of 1 mg of Chl/mL in a solution of 1 M $CaCl_2$ and 40 mM MES, pH 6.0, in darkness at 4 °C. At the times indicated, aliquots that contained 15 μg of Chl were diluted into 2 mL of the reaction mixture to assay photosynthetic O_2-evolving activity such that the final concentration of $CaCl_2$ was 7.5 mM during the assay. (B) Effect of addition of the 33-kDa protein on the O_2-evolving activity of $CaCl_2$-washed PSII preparations. PSII preparations were incubated at 1 mg of Chl/mL in 1 M $CaCl_2$ for 10 min (open symbols) or 60 min (closed symbols) to inhibit O_2-evolving activity. The incubation in 1 M $CaCl_2$ was terminated by diluting 100 μL of the particles into 2 mL of 50 mM MES, pH 6.0, which contained the amount of P_{33} (squares) or SSADP-modified P_{33} (circles) indicated for reconstitution. The rate of water oxidation was then assayed by adding 30 μg of Chl of reconstituted PSII particles to the reaction mixture. The concentration of $CaCl_2$ during the assay was 15 mM. The rate of O_2 evolution before (diamonds) and after (triangles) the $CaCl_2$ wash is indicated.

(Model UVG-54, Ultraviolet Products, Inc., San Gabriel, CA) for 4 min at 4 °C. Under these conditions the sample received about 5.5 kerg cm^{-2} s^{-1} of light.

Proteins were separated by SDS–PAGE with 10% acrylamide as described by Chua (1980). After solubilization of the membranes with 1% SDS at 37 °C for 2 h (and 70 °C for 4 min where indicated), the cross-linked proteins were purified by preparative SDS–PAGE with tube gels (1.5 cm × 14 cm) that contained 10% acrylamide (and 6 M urea where indicated). The preparative gels were sliced into disks, and where indicated, the proteins were electroeluted from the gels into a salt bridge of ammonium acetate with the Model KVEA electroeluter (International Biotechnologies, Inc.). Samples that were assayed for enzymatic activity were desalted immediately by centrifugation chromatography with Sephadex G-25 in 40 mM MES, pH 6.0. For protein analysis the ammonium acetate solution containing the electroeluted proteins was incubated at −20 °C for 10 min to precipitate the proteins with the SDS. The supernatant was removed after a 10-min centrifugation in an Eppendorf microfuge; the pellet was resuspended in 40 mM MES and desalted by centrifugation chromatography with Sephadex G-25 in 1% (w/v) SDS and 40 mM MES, pH 6.0.

Samples subjected to Western blot analyses were separated by analytical SDS–PAGE on 10% slab gels, and the proteins were transferred to nitrocellulose according to procedures previously described (Sayre et al., 1986) with the exception that 5% (w/v) dry milk was substituted for BSA in the blocking buffer. Peptide-specific antibodies against the D_1 and D_2 proteins were generated against synthetic peptides corresponding to hydrophilic sequences of the D_1 and D_2 proteins according to the procedures of Sayre et al. (1986). The amino acid sequences of the synthetic peptide antigens for the D_2 and D_1 proteins correspond to residues 98–107 and residues 333–342, respectively.

RESULTS

The duration of the 1 M $CaCl_2$ wash used to remove the P_{33} from PSII preparations affects the extent of reconstitution of O_2-evolving activity that can be attained upon readdition of the P_{33}. When the rate of O_2 evolution of PSII preparations is observed as a function of the duration of the 1 M $CaCl_2$ wash (Figure 1A), the activity decreased with a $t_{1/2}$ of about 10 min. After a 60-min incubation in 1 M $CaCl_2$ less than 10% of the activity remained. The extent of reconstitution achieved as a function of added P_{33} is shown in Figure 1B after PSII membranes have been incubated in 1 M $CaCl_2$ for 10 or 60 min. After the 60-min incubation, the P_{33} increased the rate of water oxidation from 8% to 20% of the rate observed prior to the $CaCl_2$ wash. However, when the $CaCl_2$ wash was limited to 10 min, the P_{33} was able to increase the rate of O_2-evolving activity from 51% to about 85% of the original rate.

Table I: Photosynthetic O_2-Evolving Activity of PSII Preparations at Individual Stages of the Formation of the Cross-Linked PSII Proteins

treatment	expt 1, v^a	expt 2		expt 3	
		v	%[b]	v	%
untreated PSII preparations	300	323		344	100
after a 1 M $CaCl_2$ wash	191	204			
after SSADP-P_{33} reconstitution/wash	289	293	100		
after illumination at 254 nm	167	177	59	213	62

[a] Expressed as μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. [b] Expressed as the average of experiments 1 and 2.

The P_{33} was modified covalently to contain adducts of the photoaffinity reagent SSADP in order to cross-link the P_{33} to its functional binding site. The extent of O_2-evolving activity reconstituted by SSADP-modified P_{33} is shown as circles in Figure 1B. Due to the conditions of this modification (see Experimental Procedures), reaction of SSADP with the P_{33} was limited to about five adducts. The P_{33} modified in this manner did not affect the ability of this protein to reconstitute O_2-evolving activity. When SSADP-P_{33} was added to PSII preparations that had been Ca^{2+} washed for 10 min at a ratio of 0.2 mg of SSADP-P_{33}/mg of Chl, reconstitution of more than 95% of the original activity could be achieved (Table I).

We had previously established that the extent of binding of the P_{33} to Ca^{2+}-washed PSII particles is directly proportional to the amount of reconstitution of O_2-evolving activity when the P_{33} is added at ratios of 0.02–0.4 mg of P_{33}/mg of Chl (Bowlby & Frasch, 1986). The SSADP-P_{33} was cross-linked to the proteins that compose the site of binding and reconstitution of activity in photosystem II after incubation of the SSADP-P_{33} with PSII preparations at a ratio of 0.2 mg of SSADP-P_{33}/mg of Chl following a 10-min Ca^{2+} wash. In Table I, the rate of photosynthetic water oxidation measured at each step in the preparation of the cross-linked protein complex is shown for two replications in experiments 1 and 2. In these experiments, the rate after the Ca^{2+} wash was 64% of the control. Addition of SSADP-P_{33} to this Ca^{2+}-washed preparation restored O_2 evolution to 93% of the original rate. Thus, of the functional reaction centers that remained after these treatments, 32% had incorporated the P_{33} modified to contain photoaffinity groups.

To cross-link the proteins that were nearest neighbors to the functionally bound SSADP-P_{33}, the reconstituted PSII preparation was illuminated with 254-nm light for 4 min. As shown in Table I, this illumination decreased the rate of water oxidation to 59% of the original activity. Since ultraviolet light is known to inactivate electron transfer in photosystem II directly (Holt et al., 1951), untreated PSII preparations were illuminated with 254-nm light under the same conditions that had been used to catalyze photoaffinity cross-linking. As

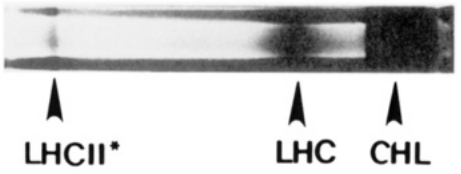

FIGURE 2: Separation of the protein constituents of cross-linked PSII particles by preparative SDS–polyacrylamide gel electrophoresis. Up to 1.2 mg of Chl was loaded on the tube gels (1.5 cm × 14 cm) after solubilization in SDS as described under Experimental Procedures. The green bands in the unstained 10% resolving gel identified as the unbound chlorophyll, the light-harvesting chlorophyll proteins (LHC), and the LHCII* protein aggregate are indicated.

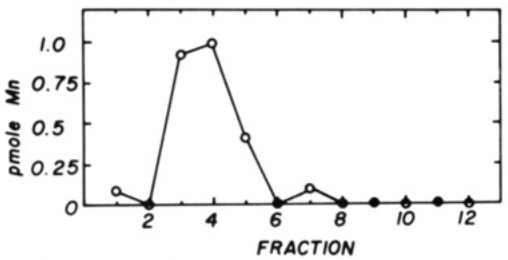

FIGURE 3: Abundance of manganese in fractions of proteins purified from cross-linked PSII particles that had been separated by preparative SDS–PAGE. Consecutive sections of the preparative gel were analyzed for manganese by neutron activation analysis.

shown in experiment 3 of Table I, the rate of water oxidation after the 254-nm illumination was 62% of the original activity. If the cross-linking reactions initiated by the 254-nm illumination in experiments 1 and 2 specifically inactivated all of the PSII reaction centers that contained bound SSADP-P_{33} (32% of the total) and 40% of the reaction centers that had not lost the unmodified P_{33} during the Ca^{2+} wash, this illumination would have decreased the rate of water oxidation to about 40% of the activity prior to illumination. Since the rates in experiments 1 and 2 were observed to be about 60% of the rate prior to the 254-nm illumination (the same loss as that of the untreated control), this suggests that the formation of covalent cross-links does not in itself inactivate electron transfer in photosystem II. Thus, about 60% of the cross-linked protein complexes made by this procedure retain the ability to catalyze the oxidation of water.

To analyze the constituents of the cross-linked complex, the membranes were solubilized with SDS and the proteins were fractionated. Preparative electrophoresis proved to be much more effective than size-exclusion chromatography in resolving the cross-linked complex from the other solubilized proteins. Figure 2 shows a preparative SDS–PAGE gel that was used to separate the protein constituents of PSII particles that had been cross-linked to the P_{33}. Green bands were visible on the gels which were identified as the 25–28-kDa light-harvesting chlorophyll (LHC) proteins and the 120-kDa aggregate of these proteins known as LHCII* (Bassi et al., 1987). The gels were sectioned such that the LHCII* band and the LHC band were in slices 2, 3, and 12–14, respectively. The abundance of manganese in the successive fractions of a preparative gel used to separate proteins of cross-linked PSII preparations is shown in Figure 3. Significant amounts of manganese were observed in fractions 3–5.

In Figure 4, a manganese-containing fraction was examined for the presence of proteins cross-linked to the P_{33} by Western blot analysis with antibody to the P_{33}. Although the fraction contained the P_{33} (lane 3), the protein appears as a high molecular mass band compared to the mobility of the P_{33} in the control (lane 1). This altered mobility resulted from

FIGURE 4: Western immunoblot probed with anti-P_{33} used to indicate the presence of proteins cross-linked to the P_{33}. Proteins were separated by SDS–PAGE, transferred to nitrocellulose as described under Experimental Procedures, challenged with antibody to the P_{33}, and then developed by horseradish peroxidase conjugated anti-IgG. Lane 1, un-cross-linked PSII particles; lane 2, proteins treated with 50 mM DTT after electroelution from fraction 4 of a preparative SDS–PAGE gel used to separate the constituents of cross-linked PSII preparations; lane 3, same as lane 2 but without DTT.

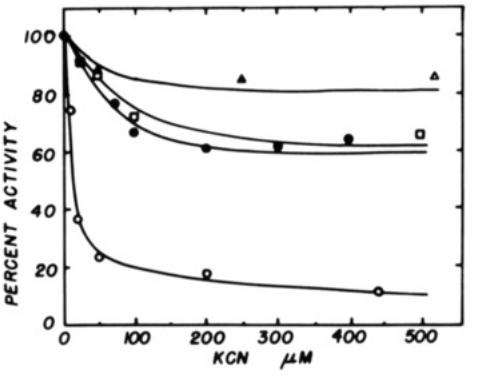

FIGURE 5: Inhibition by cyanide of (●) the catalase activity of the cross-linked complex, (○) the catalase activity from PSII preparations, (□) the catalase activity from PSII reaction center complexes, and (△) photosynthetic water oxidation from PSII preparations.

cross-links via SSADP because the addition of dithiothreitol (DTT), which will reduce the disulfide bonds of the cross-linking groups, caused the P_{33} to have the same mobility as the control (lane 2).

We have previously observed that the OEC can catalyze the conversion of H_2O_2 to O_2 by a mechanism similar to a catalase reaction (Frasch & Mei, 1987; Frasch et al., 1988). PSII preparations also have a large abundance of Fe catalase, which is mostly removed during the purification of PSII reaction center complexes (Frasch & Mei, 1987). The two reactions can be distinguished because (i) much lower concentrations of KCN inhibit the Fe catalase than the OEC and (ii) the conversion of H_2O_2 to O_2 by the OEC is activated by Ca^{2+} and Cl^- and inhibited by reagents known to inhibit water oxidation (Frasch & Mei, 1987).

The purified cross-linked complex also exhibits catalase activity. The ability of KCN to inhibit the catalase activity of the purified cross-linked complex, PSII reaction center complexes, and PSII preparations is shown in Figure 5. The same range of KCN concentrations required to inhibit photosynthetic water oxidation in PSII preparations inhibited H_2O_2-dependent O_2 evolution in PSII reaction center complexes. However, the catalase activity of PSII preparations was inhibited by KCN concentrations that were an order of magnitude lower than the concentrations required to inhibit the OEC-dependent processes. The catalase activity of the

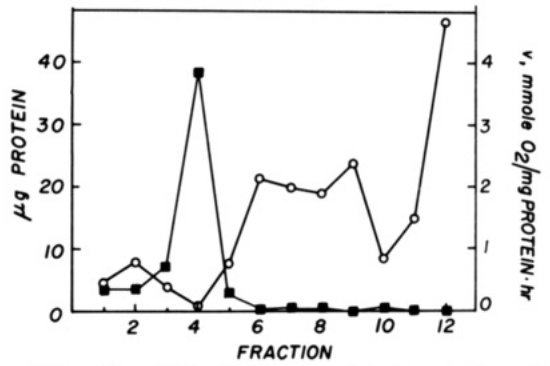

FIGURE 6: Specific activity of H_2O_2-dependent O_2 evolution catalyzed by proteins purified from cross-linked PSII particles that had been separated by preparative SDS–PAGE. The proteins were electroeluted from consecutive sections of the preparative gel as described under Experimental Procedures. The catalase activity (squares) was determined with 130 mM H_2O_2, 15 mM $CaCl_2$, 50 μM KCN, and 40 mM MES, pH 6.0. The protein content (circles) in each electroeluted fraction was determined by the bicinchoninic acid assay (Smith et al., 1985).

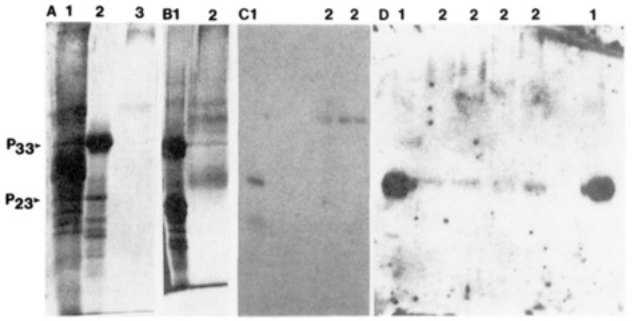

FIGURE 7: Polypeptide constituents of the cross-linked complex. Cross-linked PSII preparations were solubilized in SDS and incubated at 70 °C for 4 min to dissociate the LHCII*; then the cross-linked complex was purified by preparative SDS–PAGE with 6 M urea. The proteins were electroeluted from the section of gel enriched in the cross-linked complex and analyzed by silver-stained SDS–PAGE or by Western immunoblot analysis. (A) Silver-stained SDS–PAGE in the absence of DTT: lane 1, PSII preparations; lane 2, extrinsic membrane proteins extracted from PSII preparations by a 1 M $CaCl_2$ wash; lane 3, purified cross-linked complex. (B) Silver-stained SDS–PAGE of samples with 50 mM DTT: lane 1, extrinsic proteins from a 1 M $CaCl_2$ wash; lane 2, purified cross-linked complex. (C) Western immunoblot challenged with anti-D_2: lane 1, PSII preparations; lane 2, purified cross-linked complex with 50 mM DTT. (D) Western immunoblot challenged with anti-D_1: lanes 1 and 2 are as in (C).

cross-linked protein complex was inhibited by cyanide only at concentrations that were comparable to the OEC-dependent reactions. Thus, it is unlikely that the Fe catalase present in PSII preparations is a constituent of the cross-linked complex.

The effect of inhibitors and activators of the oxygen-evolving complex on the catalase activity of the purified cross-linked protein complex was also examined. Addition of 15 mM $CaCl_2$ to the assay increased the rate of H_2O_2-dependent O_2-evolving activity by more than 3-fold. Both Ca^{2+} and Cl^- were responsible for the rate increase since the addition of 30 mM NaCl also increased the rate but only by about 30%. These ions have been shown to be required by the oxygen-evolving complex to catalyze both water oxidation (Ghanotakis et al., 1984; Izawa et al., 1969) and H_2O_2-dependent O_2 evolution (Frasch & Mei, 1987). The dependence of the rate on Ca^{2+} is supported further by the observation that the reaction is completely inhibited by 5 mM La^{3+} which is known to be a competitive inhibitor versus Ca^{2+} for photosynthetic (Ghanotakis et al., 1985) and H_2O_2-dependent (Frasch et al., 1987) O_2 evolution. Hydroxylamine, which will inactivate water oxidation by releasing the manganese of the OEC (Cheniae & Martin, 1970), also abolished the rate of H_2O_2-dependent O_2 evolution catalyzed by the cross-linked complex when added at a concentration of 200 μM. These results strongly suggest that the manganese is bound to the cross-linked proteins in a manner which allows S-state transitions to occur.

In Figure 6, the specific activity with which each fraction electroeluted from a preparative SDS–PAGE gel catalyzed the conversion of H_2O_2 to O_2 (squares) was used as a measure of the purity of the cross-linked complex. The protein concentration of these successive fractions (circles) was determined by the BCA protein assay (Smith et al., 1985), which is not susceptible to interference by detergent. As with Figure 3, the gel had been sectioned such that the LHCII* and the LHC proteins were in fractions 2, 3, and 12–14, respectively. Fractions 5–10 contained proteins of 47–30 kDa that were not cross-linked. The specific activity of H_2O_2-dependent O_2 evolution catalyzed by the electroeluted samples is also shown in Figure 6 (squares). Although the total catalase activity observed (per 40 μL of sample) was higher in fraction 3, fraction 4 had the highest specific activity. Fraction 4 was found to contain 0.344 ± 0.013 μg of manganese/180 μg of protein.

The polypeptide constituents of the complex were analyzed by using the cross-linked complex that had been purified by preparative electrophoresis with two modifications known to deplete the preparation of the LHCII* aggregate and the D_1D_2 heterodimer: (i) the SDS-solubilized PSII preparations containing the cross-linked proteins were briefly incubated at 70 °C and (ii) the preparative gels contained 6 M urea. Without the LHCII* as a landmark, it is difficult to section the preparative gel to optimize for purity of the cross-linked complex. A silver-stained SDS–PAGE gel of the complex after such purification is shown in Figure 7A, lane 3. A high molecular mass band predominates, although a 45–50-kDa protein is also visible. The high molecular mass band was determined to contain the P_{33} by immunoblot analysis as described above.

The addition of DTT to the purified cross-linked sample (Figure 7B, lane 2) did not affect the amount of protein present in the lower molecular mass band, and thus, it is unlikely that this protein has cross-linked to the P_{33}. However, the DTT treatment caused the high molecular mass band to be replaced by four bands at lower molecular masses. Although the intensity of three of the four new bands is about the same, the abundance of the protein that runs just above the P_{33} is significantly lower. The diffuse staining of these bands relative to the bands in the lane containing PSII particles may be due, in part, to the heterogeneous formation of the covalent adducts formed by the photoaffinity group of SSADP.

To probe the identity of the proteins cross-linked to the P_{33}, the disulfide bonds of the purified cross-linked complex were reduced; then the proteins were resolved by SDS–PAGE and transferred to nitrocellulose for Western immunoblot analysis. The immunoblot of the cross-linked proteins challenged with antibodies of D_2 shown in Figure 7C indicates the presence of D_2 as one of the cross-linked proteins. Evidently, the covalent adducts formed upon photoaffinity cross-linking alter the electrophoretic mobility of this protein. Western blot analysis also identified the D_1 protein as a component of the cross-linked complex (Figure 7D). Although the electrophoretic mobility of D_1 is not altered as a result of cross-linking, the presence of these adducts decreases the immunoreactivity of D_1 relative to D_2 when compared to the level of reactivity of the corresponding proteins in PSII particles. This apparent

decrease in reactivity may be due to the fact that the D_1 antigen is localized to a polypeptide segment of D_1 that is exposed to the luminal side of the membrane (Sayre et al., 1986), the same surface to which the P_{33} binds.

The molecular weights of the P_{33}, D_1, and D_2 proteins have been determined from the sequences of the proteins to be 26 663, 38 950, and 39 563, respectively (Oh-oka et al., 1986; Tyagi et al., 1987; Zurawski et al., 1982; Alt et al., 1984; Holschuh et al., 1984). On the basis of a 1:1 ratio of these proteins, the total M_r of the cross-linked complex is about 1.05×10^5. If no other proteins comprise the complex, the ratio of 0.34 μg of manganese/180 μg of protein translates to a mole ratio of 3.6 Mn/protein. If the fourth protein of the cross-linked complex is also present with a unit stoichiometry to the other proteins or if the complex contains two copies of the P_{33}, the ratio of Mn/protein increases to about 4.5.

Discussion

The experiments described here show that the P_{33} can be cross-linked to the proteins that compose its binding site in Ca^{2+}-washed PSII preparations such that the cross-linked proteins can oxidize water. The cross-linked complex thus formed can be solubilized with SDS and purified by electrophoresis. The complex is composed of the P_{33} cross-linked primarily to the D_1 and D_2 proteins. After solubilization from the membrane and purification by electrophoresis, the complex retains bound manganese and can catalyze the conversion of H_2O_2 to O_2.

Hydrogen peroxide has been found to serve as an alternate substrate of the OEC for the production of molecular oxygen (Velthuys & Kok, 1978; Frasch & Mei, 1987; Frasch et al., 1987; Mano et al., 1987). In these reactions, H_2O_2 first causes a $2e^-$ oxidation of the S_0 state to the S_2 state. A second H_2O_2 then is oxidized to O_2 by the S_2 state. A similar reaction occurs via a cycle between the S_{-1} and S_1 states. We have recently found that the mechanism of these reactions has close similarities to that of catalase (Frasch et al., 1988). Reagents that are known to inhibit photosynthetic O_2 evolution decrease the activity of these H_2O_2-dependent reactions with the same effectiveness. Although both the S_{-1}/S_1 and S_0/S_2 reactions require Ca^{2+}, only the latter reaction is dependent on Cl^- (Frasch et al., 1987). The observation that inhibitors and activators of the OEC have the same effects on the catalase activity of the cross-linked complex indicates that the manganese must be bound to this complex in a form in which some S-state transitions can occur. Since Cl^- causes a small increase in the activity of the cross-linked complex, at least a portion of the complexes can cycle between the S_0 and S_2 states.

The manganese content of the cross-linked sample approaches a ratio of 1.9 μg of Mn/mg of protein when the specific activity of the H_2O_2 assay is maximal. The precision of the BCA protein assay is high and is not susceptible to interference by detergent. However, this protein assay is similar to others in that it can introduce systematic error in the measurement of the amount of unknown protein determined from a standard curve with a different protein.

A mole ratio of about 4 Mn/cross-linked complex is calculated if two assumptions are made: (i) that the samples used for the manganese/protein ratio need not be corrected for purity and (ii) that the polypeptides of the cross-linked complex are present in equal proportions. Measurements of the stoichiometry of the P_{33}/PSII reaction center yield results that vary between 1 (Murata et al., 1983) and 2 (Åkerlund, 1983; Cammarata et al., 1984). If the cross-linked complex consists of two P_{33} and a single copy of each of the other three proteins, the mole ratio of manganese to protein will exceed 5. However, the size of the band corresponding to the unknown protein of the cross-linked complex (Figure 7B, lane 2) is significantly smaller than the D_1, D_2, and P_{33} bands. Thus, this protein may be cross-linked to the P_{33} in only a fraction of the complexes, which allows for the possibility of the presence of two copies of the P_{33} per complex.

A number of observations support the hypothesis that the D_1 and D_2 proteins contain the prosthetic groups of the reducing side of PSII [for a review, see Michel and Deisenhofer (1988)]. The electron-transfer components of the oxidizing side of photosystem II have been suggested to be associated with the D_1 and D_2 proteins as well. Site-directed mutagenesis has been used to identify tyrosine-161 of D_1 and tyrosine-160 of D_2 as Z and D, respectively (Barry & Babcock, 1987; Debus et al., 1988a,b). The D_1 protein has been implicated to have a role in binding the OEC manganese from the observation that this protein is altered in the LF-1 mutant of *Scenedesmus* (Metz et al., 1980, 1985), which lacks bound manganese and does not have a functional OEC.

It is noteworthy that the D_1 protein was identified in Figure 5 by an antibody made specifically to residues 333–342 in the sequence of D_1. The maturation of D_1 involves the proteolytic cleavage of a 2-kDa peptide from the C-terminus (Grebanier et al., 1978; Marder et al., 1984). Diner et al. (1988b) have recently demonstrated that antibodies directed against the final 14 amino acid residues (340–353) of the protein will recognize the D_1 protein from the LF-1 mutant but not the wild-type protein. The synthetic peptide antigen used by Diner et al. (1988b) overlaps the antigen used in this study by three amino acids (residues 340–342). This suggests that proteolytic cleavage must occur at a site bound by these two antigens. On the basis of the immunological results and the conservation of the amino acid sequences between species in this region of D_1 (Erickson et al., 1985), we suggest that the site of cleavage must be located between amino acid residues 344–345. In support of this hypothesis we note that the D_1 protein from *Euglena gracilis* ends at residue 344 (Keller & Stutz, 1984). Failure to correctly process the D_1 protein results in the inability to bind functional manganese and oxidize water. However, since the LF-1 is the result of a processing mutation, the observed lack of manganese is not necessarily the result of the inability of the metal to bind to the D_1 protein.

The evidence presented here indicates that at least one of the proteins which has become cross-linked to the P_{33} binds the manganese of the OEC. Although the P_{33} has been demonstrated to be closely associated with the process of water oxidation (Åkerlung & Jansson, 1981; Hunziker et al., 1987), the ability of P_{33}-depleted preparations to form the multiline signal (Styring et al., 1987; Miller et al., 1987) and advance from S_0 to S_3 with normal kinetics (Dekker et al., 1984; Miyao et al., 1987a) indicates that the P_{33} does not form ligands to the functional manganese directly during these S-state transitions. Due to the low abundance of the unidentified cross-linked protein relative to the P_{33}, D_1, and D_2 proteins, the D_1 and D_2 proteins are the most likely candidates to contain the binding site(s) for the manganese.

Although the cross-linked complex is capable of some S-state transitions, we have not observed measurable rates of photosynthetic water oxidation after the membranes have been solubilized with SDS. This may result from the loss of an electron-transfer group upon solubilization. Diner et al. (1988a) have observed the loss of quinone from the Q_A site during the purification of PSII reaction center particles from *Chlamydomonas reinhardii* by solubilization in Triton X-100. Alternatively, the cross-linked complex may require additional

proteins that are present in PSII reaction center preparations in order to form the S_3 state and/or the S_4 state.

Registry No. Mn, 7439-96-5; Ca, 7440-70-2; Cl^-, 16887-00-6; H_2O, 7732-18-5; O_2, 7782-44-7; catalase, 9001-05-2.

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Effect of Monoclonal Antibodies on the Properties of Smooth Muscle Myosin†

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ABSTRACT: Monoclonal antibodies were generated against turkey gizzard myosin, and their effects on some of the properties of myosin were assayed. Ca^{2+}- and Mg^{2+}-ATPase activities of myosin were enhanced by the anti-subfragment 2 antibodies at low ionic strength (i.e., with 10S myosin). Tryptic fragments of an anti-S2 IgM also activated these activities. Antibodies directed against subfragment 1 and light meromyosin had no effect. The Mg^{2+}-ATPase activity of heavy meromyosin also was activated by an anti-S2 antibody. Actin-activated ATPase activity of phosphorylated myosin was enhanced by the anti-S2 IgM fragments at low $MgCl_2$ concentrations. This increase was reflected by a 5-fold increase in V_{max} and a slight decrease in the apparent dissociation constant for actin. The actin-activated ATPase of dephosphorylated myosin was not affected by intact anti-S2 antibody or its fragments. The rates of phosphorylation and dephosphorylation of the 20 000-dalton light chains were increased by interaction of myosin with anti-S2 antibody. Limited proteolysis of myosin was used as a conformational probe. Interaction of anti-S2 antibody with 10S myosin increased the extent of cleavage at the S1–S2 junction. Proteolysis of 6S myosin was rapid and was not influenced by anti-S2 antibody. Our interpretation of these results is that interaction of the anti-S2 antibodies with myosin alters the conformation in the S2 region and this in turn modifies some of the properties of myosin. This is consistent with the hypothesis that the S2 region of smooth muscle myosin is a determinant of its biological properties.

Phosphorylation of myosin is an important regulatory component of smooth muscle. Although the relationship between the level of developed tension and phosphorylation is complex, it is accepted that the phosphorylation of myosin is required for contraction (Hartshorne, 1987). The sites of phosphorylation are the two 20 000-dalton light chains of myosin, and under most circumstances, the serine-19 residues are phosphorylated. The phosphorylation reaction is catalyzed by the Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK),[1] and dephosphorylation is achieved via a phosphatase, whose identity is not established.

The critical biochemical change elicited by phosphorylation is an increase of actin-activated ATPase activity, and this event is thought to reflect an increased rate of cross-bridge cycling in the intact tissue. Phosphorylation of the light chains thus initiates a conformational change that is transmitted to the myosin heads (S1) and results in modification of the active site(s). Details of the molecular events involved in this process are not established, but some interesting preliminary results have been obtained. These stem from the discovery that smooth muscle myosin can exist in two conformations, i.e., the extended (6S) and folded (10S) states (Suzuki et al., 1982), and that the conformation correlates with enzymatic activity (Ikebe et al., 1983). The simple hypothesis was developed (Ikebe et al., 1983) that the conformation of myosin determined enzymatic properties and that the critical conformational changes occurred as part of the 10S–6S transition. [Since myosin exists in thick filaments in both relaxed and contracting smooth muscle (Somlyo et al., 1981), it is unlikely that the entire folding transition is allowed in intact muscle.] According to this scenario, the role of phosphorylation is to alter conformation that in turn influences ATPase properties, and in support of this was the observation that under appropriate ionic conditions phosphorylation will convert 10S to 6S myosin (Ikebe et al., 1983; Craig et al., 1983; Onishi et al., 1983; Trybus & Lowey, 1984). The 6S–10S transition, however, could be a composite of several more subtle changes, and the problem is to identify those changes that affect biological properties. Using limited proteolysis as a conformational probe, it was shown that two sites are altered during the 10S–6S transition. These were designated sites A and B (Ikebe & Hartshorne, 1985a,1986) and are located at, or close to, the actin binding site and the S1–S2 junction, respectively. In the 6S state, site B is accessible to proteolysis (Ikebe & Hartshorne, 1984; Onishi & Watanabe, 1984), and the idea developed that changes in flexibility at the S1–S2 junction altered the mobility of the myosin heads. In the inactive state (10S equivalent), it was suggested that the heads are con-

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[1] Abbreviations: MLCK, myosin light chain kinase; HMM, heavy meromyosin; LMM, light meromyosin; S1, heavy meromyosin subfragment 1; S2, heavy meromyosin subfragment 2; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ELISA, enzyme-linked immunosorbent assay; IMDM, Iscove's modified Dulbecco's medium; HAT, hypoxanthine–aminopterin–thymidine; FBS, fetal bovine serum; NFDM, nonfat dry milk.

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