Oxidation of Alcohols Catalyzed by the Oxygen-Evolving Complex†

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ABSTRACT: The effects of alcohols on photosystem II reaction center complex preparations were examined. The order of effectiveness of alcohols to inhibit photosynthetic O_2 evolution was propargyl alcohol > glycerol > ethanol ≫ ethylene glycol. Inhibition by alcohols of H_2O_2-dependent O_2 evolution catalyzed by the oxygen-evolving complex (OEC) had the same concentration dependence as observed for the inhibition of photosynthetic O_2 evolution with the exception of glycerol, which inhibited H_2O_2-dependent O_2 evolution most effectively. Glycerol and propargyl alcohol were competitive inhibitors of H_2O_2-dependent O_2 evolution with inhibition constants of 12.9 mM and 1.16 M, respectively. Low concentrations of alcohols (<200 mM) in the presence of H_2O_2 inhibited the OEC without causing the release of manganese. However, inactivation and loss of manganese can occur at higher concentrations of alcohol. The oxygen-evolving complex, in the presence of H_2O_2, catalyzes the oxidation of ethanol, glycerol, and propargyl alcohol to the respective aldehydes. A mechanism for this alcohol oxidase reaction catalyzed by the OEC is proposed.

The oxygen-evolving complex (OEC)[1] uses the oxidizing potential generated from photosystem II (PSII) to drive the oxidation of water to molecular oxygen. The four bound manganese that are central to this process (Cheniae & Martin, 1972) become oxidized in a manner that allows the sequential loss of single electrons from the complex. As a result of the loss of each electron, the OEC advances sequentially through stable or semistable states known as S states (Kok et al., 1970; Forbush et al., 1971). Molecular oxygen is evolved only after the formation of the S_4 state.

From the observation of flash-induced yields of O_2 from thylakoids in the presence of H_2O_2, Velthuys and Kok (1978) hypothesized that hydrogen peroxide can serve as a substrate for the OEC. We have found recently that the OEC can use H_2O_2 as an alternate substrate without light-generated oxidizing equivalents (Frasch & Mei, 1987a,b). In this reaction, O_2 is evolved by interconversion of the S states without the participation of charge separation from the reaction center. These findings have been confirmed by experiments which show $^{18}O_2$ evolution from PSII preparations in the presence of $H_2{}^{18}O_2$ (Mano et al., 1987).

The OEC appears to convert H_2O_2 to O_2 via a two-step mechanism in a manner similar to the mechanism of catalase (Frasch & Mei, 1987b). The first hydrogen peroxide causes a two-electron oxidation of the OEC which reduces H_2O_2 to form water. The OEC then oxidizes a second H_2O_2 by a two-electron step and evolves O_2. The OEC can catalyze this reaction via an S_0/S_2 state cycle which requires chloride and calcium. This reaction is inhibited by reagents known to interact specifically with the S_2 state. The reaction can be catalyzed by S_1/S_{-1} state transitions as well. A kinetic study of the S_1-dependent H_2O_2 reaction revealed an equilibrium ordered mechanism in which the binding of Ca(II) to the OEC must preceed the binding of H_2O_2 (Frasch & Mei, 1987b). Consequently, Ca(II) cannot dissociate once H_2O_2 has added or during any part of the catalytic cycle until H_2O_2 is free to add again.

The manganese in the OEC gives rise to two low-temperature EPR signals in the S_2 state known as the multiline signal (Dismukes & Siderer, 1980; Brudvig et al., 1983; Zimmermann & Rutherford, 1984) and the $g = 4.1$ signal (Zimmermann & Rutherford, 1984; Casey & Sauer, 1984). Alcohols have been shown to increase the intensity of the multiline signal at the expense of the $g = 4.1$ signal (Zimmermann & Rutherford, 1986). Ethanol has been found to decrease the line width of the multiline signal as well, which suggests that alcohols may interact directly with the manganese in the S_2 state (Hansson et al., 1987).

In this study we examine the effect of alcohols on the OEC. By using aldehyde dehydrogenase as a coupled assay, we have observed that the OEC, in the presence of H_2O_2, can catalyze the formation of aldehyde from ethanol, glycerol, or propargyl alcohol. A mechanism for the alcohol oxidase activity catalyzed by the OEC is proposed.

Materials and Methods

Reaction Center Complex Preparations and Assays for O_2 Evolution. Photosystem II reaction center complex preparations were prepared as described by Ghanotakis and Yocum (1986), and rates of oxygen evolution were measured with a Clark-type O_2 electrode. Assays for oxygen evolution from H_2O_2 in darkness and photosynthetic O_2 evolution were measured as described by Frasch and Mei (1987a). The presence of the highest concentrations (800 mM) of alcohols used in this study affected neither the sensitivity of the O_2 electrode response to the level of air saturation nor the response time of the electrode. Thus, under the conditions employed here, alcohols did not present a diffusion barrier for O_2 migration and reaction at the electrode.

To remove the manganese from the oxygen-evolving complex, reaction center complex preparations (100 μg of Chl/mL) were suspended in 0.8 M Tris, pH 8.3, and illuminated at 4 °C for 30 min. The membranes were then removed from the solution by centrifugation in a microfuge and were resuspended

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[1] Abbreviations: PSII, photosystem II; OEC, oxygen-evolving complex; MES, 4-morpholineethanesulfonic acid; Chl, chlorophyll; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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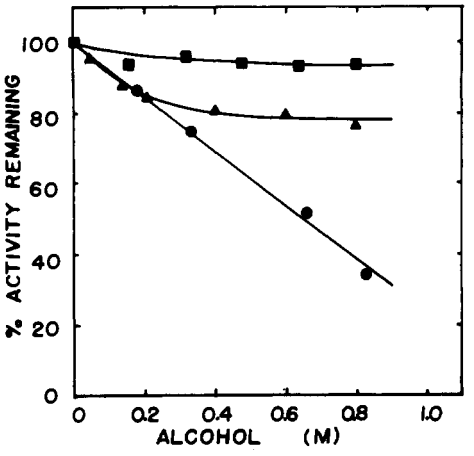

FIGURE 1: Dependence of the rate of photosynthetic O_2 evolution on the concentration of ethanol (■), glycerol (▲), or propargyl alcohol (●). The rate in the absence of inhibitor was 1081 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

in 50 mM MES, pH 6.0, with 20 mM EDTA for 15 min. The EDTA solution was removed by centrifugation, and the membranes were washed once in the MES, pH 6.0 buffer before final resuspension in the same buffer. The abundance of hexaquamanganese was determined by the signal intensity of the six-line EPR signal on a Bruker ER200E EPR. The instrumental conditions were as follows: microwave power, 25 mW; gain, 5×10^5; time constant, 100 ms. The signal intensity of the six-line EPR signal was determined to be unaffected by the ligand environment created by the reagents present in the assay. These reagents include the concentrations of alcohols and/or H_2O_2 that were used in Figure 3.

End-Point Assays for the Formation of Aldehyde. Just prior to use, the alcohols were distilled to remove contaminating aldehydes. The ability of PSII reaction center preparations to catalyze the formation of aldehydes from alcohols was determined by incubation of reaction centers (7.2 μg of Chl/mL) in 130 mM H_2O_2, 200 mM alcohol, 50 μM KCN, 0.1 mM EDTA, and 50 mM MES, pH 6.0 buffer for 15 min in darkness at 25 °C. The amount of aldehyde formed by the reaction center preparations was determined by enzymatic assay in 100 mM K^+/HEPES, pH 8.0, at 25 °C as described by Rendina and Cleland (1981). The assay contained 2 mM NAD^+, 100 mM KCl, 10 mM dithiothreitol, and 50 units of aldehyde dehydrogenase in a 2-mL volume. The reaction was allowed to go to completion (1 h), and the net change at 340 nm was monitored spectrophotometrically in cuvettes with a 1-cm path length. Concentrations were calculated with A_{340} = 6.2 mM^{-1} cm^{-1} for NADH.

Analysis of Dead-End Inhibition Kinetic Data. The initial velocity data were expressed as double-reciprocal plots and slope replots, used to graphically determine the kinetic constants, to check the linearity of the curves, and to determine the pattern of the plots. Analysis of the kinetic constants of Figure 3 was then made by use of the COMPO computer program of Cleland (1967) which was translated into BASIC for use with a personal computer. This program fits a linear competitive inhibition pattern to eq 1, where S is the substrate

$$v = \frac{VS}{K_M(1 + I/K_{is}) + S} \quad (1)$$

concentration, I is the concentration of inhibitor, v is the observed rate at a given S and I, V is the maximal velocity of the enzyme, K_M is the Michaelis constant, and K_{is} is the inhibition constant of the slopes. Fitting the data to this equation provided the most accurate estimate of the values for the kinetic constants with standard errors.

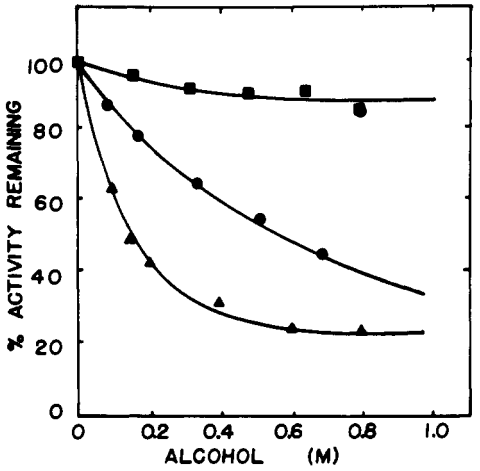

FIGURE 2: Dependence of the rate of O_2 evolution from H_2O_2 on the concentration of ethanol (■), glycerol (▲), or propargyl alcohol (●). Assays were carried out in darkness and contained PSII reaction center preparations (4 μg of Chl), 130 mM H_2O_2, 50 μM KCN, 10 mM $CaCl_2$, and 40 mM MES, pH 6, in 2-mL final volume. The rate in the absence of inhibitor was 952 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}.

RESULTS

Alcohols were found to inhibit the oxygen-evolving complex as shown in Figure 1, where the extent of inhibition of photosynthetic oxygen evolution is plotted as a function of the concentration of alcohol. The order of effectiveness of these alcohols as inhibitors of photosynthetic O_2 evolution is propargyl alcohol > glycerol > ethanol ≫ ethylene glycol. Ethylene glycol did not inhibit photosynthetic O_2 evolution (data not shown). Concentrations of 800 mM ethanol (~10% v/v) and 800 mM glycerol (~7% v/v) decreased the activity by only 10% and 20%, respectively. However, 800 mM propargyl alcohol inhibited photosynthetic O_2 evolving activity by 70%.

The effect of alcohols on the rate of O_2 evolution from H_2O_2 in darkness catalyzed by the OEC is shown in Figure 2. Ethanol and propargyl alcohol inhibited this reaction with the same concentration dependence as observed for photosynthetic O_2 evolution while ethylene glycol had no effect. However, glycerol was a more effective inhibitor of H_2O_2-dependent O_2 evolution than of photosynthetic O_2 evolution such that 60% of the H_2O_2-dependent activity was inhibited by the addition of 200 mM glycerol. Both glycerol and propargyl alcohol showed linear competitive inhibition versus hydrogen peroxide and had K_{is} values of 12.9 mM and 1.16 M, respectively. These results suggest that alcohols displace H_2O_2 from the catalytic site of the OEC.

The extent to which manganese is released from the OEC by H_2O_2 and alcohols was determined by measuring the signal intensity of the Mn(II) six-line EPR signal in PSII reaction centers after the addition of these reagents (Figure 3). The total amount of manganese in the sample was determined by the addition of 10 mM NH_2OH. The addition of 130 mM hydrogen peroxide did not cause the release of manganese from the reaction center preparation. As illustrated in spectra C and E of Figure 3, the six-line signal was also absent in the H_2O_2-containing sample after addition of either 200 mM ethanol or 200 mM propargyl alcohol. However, about 25% of the manganese present in these preparations was released by 200 mM glycerol with 130 mM H_2O_2 (Figure 3D). Although the extent of release of manganese and the loss of photosynthetic O_2-evolving capacity induced by 200 mM glycerol are approximately the same, this concentration of

Table I: Oxidation of Propargyl Alcohol to Aldehyde by PSII Reaction Center Complex Preparations in the Presence of Hydrogen Peroxide[a]

RC complexes (7.4 μg/mL)	propargyl alcohol (200 mM)	H_2O_2 (130 mM)	NH_2OH (5 mM)	A_{340}[b]	ΔA_{340}	nmol of RCHO formed
+	−	−	−	0.164		
+	+	−	−	0.422	0[c]	0
+	+	+	−	0.913	0.419	159
+	+	+	+	0.379	0	0
Tris/EDTA washed	+	+	−	0.407	0	0

[a] Each assay contained 50 μM KCN, 0.1 mM EDTA, and 50 mM MES, pH 6.0, in 2 mL and was incubated at 25 °C for 15 min in darkness. Analysis of aldehyde was determined subsequently by a coupled assay with aldehyde dehydrogenase as described under Materials and Methods. The absorbance of the samples was measured in the presence of the components shown above and the reagents of the coupled assay. [b] Absorbance at 340 nm when 40 mM MES, pH 6.0, was used as a reference. [c] Absorbance increases were determined relative to this sample.

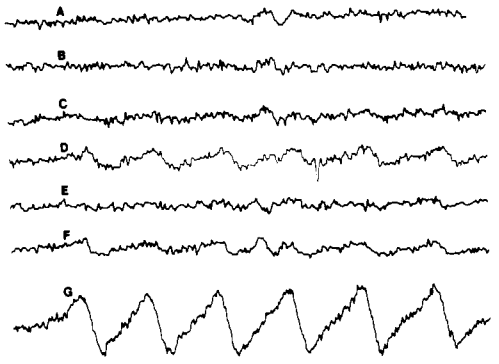

FIGURE 3: Extent of release of Mn from the OEC which results from the addition of alcohol and/or H_2O_2 in darkness as measured by the signal intensity of the Mn six-line EPR signal. The reaction center complex preparations (0.4 mg of Chl/mL) were suspended in 40 mM MES, pH 6.0, 10 mM $CaCl_2$, and (A) no additions, (B) 130 mM H_2O_2, (C) 130 mM H_2O_2 and 200 mM ethanol, (D) 130 mM H_2O_2 and 200 mM glycerol, (E) 130 mM H_2O_2 and 200 mM propargyl alcohol, (F) 130 mM H_2O_2 and 300 mM propargyl alcohol, and (G) 10 mM NH_2OH.

glycerol caused the loss of 80% of the H_2O_2-dependent O_2-evolving activity. Higher concentrations of propargyl alcohol (300 mM, Figure 3F) with H_2O_2 also caused the release of about 17% of the manganese from the OEC and inhibited H_2O_2-dependent O_2 evolution by 40%.

The EPR experiments shown in Figure 3 represent the loss of manganese induced over the period of about 15 min, whereas the data from Figures 1 and 2 were obtained by continuous assay in approximately 30 s. Thus, the EPR experiments provide an upper limit for estimates of the amount of manganese released by the addition of H_2O_2 and alcohol relative to the extent of inhibition of the activity observed in Figures 1 and 2. These results suggest that low concentrations of alcohol (>200 mM) inhibit the OEC reversibly, while high concentrations (>200 mM) in the presence of H_2O_2 can cause inactivation and release of manganese.

A coupled assay was used to determine if aldehyde was formed from propargyl alcohol by PSII complex preparations in the presence of H_2O_2. The coupled assay consumes aldehyde in direct proportion to the amount of NADH formed, which is measured by the aborbance at 340 nm. As shown in Table I, when 40 mM MES, pH 6.0, is used as the reference, the reaction center complexes and the coupled enzyme system contributed an absorbance of 0.164 at 340 nm. An absorbance of 0.422 was observed upon addition of propargyl alcohol to the assay. This increase in absorbance (ΔA_{340} = 0.258) indicated that approximately 83 nmol of aldehyde contaminated the alcohol. When both H_2O_2 and alcohol were present, the absorbance increased to 0.913. This increase was not observed if 5 mM NH_2OH was added to the assay or if Tris/EDTA-washed reaction center preparations were used. The active preparation showed a net increase of 0.491 absorbance unit from the reaction which lacked H_2O_2. Thus, the core preparation catalyzed the formation of about 159 nmol of propargyl aldehyde in the presence of H_2O_2.

Table II: Comparison of Alcohols as Substrates of the Oxygen-Evolving Complex for Aldehyde Formation in the Presence of H_2O_2[a]

alcohol	ΔA_{340}	nmol of RCHO formed	mol of RCHO/ mol of OEC[b]	μmol of RCHO (mg of Chl)$^{-1}$ h^{-1}
ethanol	0.156	50.3	349	28.0
glycerol	0.218	70.2	488	39.1
propargyl alcohol	0.492	158.7	1102	88.2

[a] Each assay contained PSII reaction center complexes (7.2 μg of Chl/mL/mL) in 130 mM H_2O_2, 200 mM alcohol, 50 μM KCN, 0.1 mM EDTA, and 50 mM MES, pH 6.0, in 2 mL and was incubated at 25 °C for 15 min in darkness. Analysis of aldehyde was determined subsequently by a coupled assay with aldehyde dehydrogenase as described under Materials and Methods and in Table I. [b] Calculated with the assumption of 50 Chl/OEC as per Ghanotakis and Yocum (1986).

The results of Table II compare the ability of the reaction center complex preparations to catalyze the oxidation of ethanol, glycerol, and propargyl alcohol to the respective aldehydes. In all cases, the oxidation of alcohols was abolished when the OEC was inactivated by a Tris/EDTA wash prior to assay (data not shown). Multiple turnovers of the enzyme were observed for each alcohol used as a substrate. For example, the OEC catalyzed more than 1100 turnovers with propargyl alcohol over the duration of the assay.

The assay was carried out as an end-point determination and cannot provide the initial rates of these reactions. However, the rates shown in Table II, which were calculated for this 15-min time point, provide a lower limit for the true rate. For these alcohols, the rates observed correspond to about 5–20% of the initial rate of H_2O_2-dependent O_2-evolving activity with 130 mM H_2O_2. The relative amounts of aldehyde formed when the reaction center complexes were incubated with 200 mM concentrations of each alcohol suggest that the order of effectiveness of the alcohols as substrates is propargyl alcohol > glycerol > ethanol. The superiority with which propargyl alcohol ($HCCCH_2OH$) acts as a reductant for the OEC relative to the other alcohols probably results from an inductive effect of the acetylenic group. This may also explain why propargyl alcohol inhibits O_2 evolution more effectively without causing the release of manganese from the OEC.

Discussion

Evidence presented here shows that alcohols can bind to the active site of the OEC, which includes (i) inhibition of photosynthetic O_2 evolution by ethanol, glycerol, and propargyl alcohol; (ii) linear competitive inhibition of H_2O_2-dependent O_2 evolution by glycerol and propargyl alcohol; and (iii) oxidation by the OEC of the alcohols to aldehydes in the presence

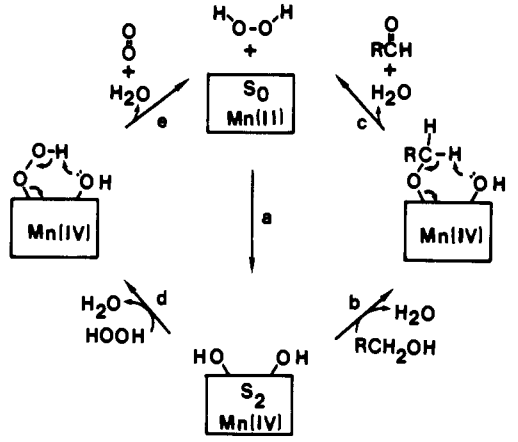

FIGURE 4: Proposed mechanism for the oxidation of H_2O_2 or alcohol catalyzed by the oxygen-evolving complex. The reactions represent an interaction of the substrates with the four manganese in the OEC although the oxidation state of one manganese is indicated.

of H_2O_2. A close interaction between alcohols and the manganese of the OEC is also supported by the alcohol-induced decrease of the $g = 4.1$ EPR signal and the proportional increase in the multiline EPR signal (Zimmerman & Rutherford, 1986). The decrease in the hyperfine line widths of the multiline EPR signal observed upon addition of ethanol (Aasa et al., 1987) suggests that alcohols coordinate with the manganese in the S_2 state.

While glycerol or ethanol decrease the amplitude of the $g = 4.1$ EPR signal, these alcohols affect the power saturation of the temperature EPR signals differently (Zimmerman & Rutherford, 1984, 1986). Evidence presented here shows that glycerol was more effective than ethanol as (i) an inhibitor of photosynthetic and H_2O_2-dependent O_2 evolution and (ii) as a substrate for the formation of aldehyde. Thus, alcohols might be expected to increase the rate of decay of the multiline EPR signal upon warming the sample because they reduce the manganese upon formation of aldehyde.

The requirement and sensitivity of H_2O_2-dependent O_2 evolution for S_2 state specific activators and inhibitors, respectively, which can be altered when the initial abundance of the S states is changed by flashes of light, indicate that this reaction can occur via a cycle between the S_0 and S_2 states (Frasch & Mei, 1987a,b). Recent evidence using $H_2{}^{18}O_2$ suggests that H_2O is displaced from the active site of the OEC by the H_2O_2 reactions (Mano et al., 1987). To account for the observed effects of H_2O_2 and alcohol on the S_2 state, we propose the mechanism shown in Figure 4 for the S_0/S_2 cycle. First, in step a, H_2O_2 binds to the S_0 state and causes a two-electron oxidation of the OEC such that one of the manganese exists as a formal Mn(IV). This results in the formation of an S_2 state which contains two hydroxides bound to the manganese cluster. Second, in steps b and c, the hydroxyl group of the alcohol displaces a coordinated hydroxide which results in the release of water. The reaction then proceeds via the abstraction of a proton at C-1 of the alcohol and subsequent two-electron flow to the manganese. In the absence of alcohol, a second H_2O_2 would displace the coordinated hydroxide, and the reaction would proceed by a two-electron step to yield O_2 as shown (steps d and e).

This mechanism is very similar to the model proposed by Schonbaum and Chance (1976) for the interaction of H_2O_2 with Fe-catalase. A Mn-catalase known as pseudocatalase has been isolated from *Lactobacillus plantarum* (Kono & Fridovich, 1983a). This enzyme contains two atoms of manganese per subunit (Beyer & Fridovich, 1985), which undergo valence changes at the active site during catalysis (Kono & Fridovich, 1983b).

This model does not attempt to describe all of the oxidation states or ligands of the four manganeses in the OEC. The low-temperature EPR spectra provide evidence that a formal Mn(IV) exists in the S_2 state (Dismukes & Siderer, 1981; Hansson et al., 1982; Brudvig et al., 1986). This corroborates the findings of a variety of physical measurements (Dekker et al., 1984; Gooden et al., 1984; Srinivasan & Sharp, 1986a,b). The NMR proton relaxation enhancement studies of Srinivasan and Sharp (1986a,b) also suggest that Mn(II) is formed upon generation of the S_0 state. The model suggests that the S_2 state formed by H_2O_2 contains two hydroxides coordinated to manganese in the cluster. Apparently, a variety of reagents can coordinate to the manganese in the S_2 state which include water (Hansson et al., 1986), alcohols (Hansson et al., 1987), and ammonia (Beck & Brudvig, 1986).

The alcohols examined in this study have the same relative effectiveness for oxidation to aldehydes as they have as inhibitors of photosynthetic O_2 evolution. However, glycerol is a more effective inhibitor of H_2O_2-dependent O_2 evolution than propargyl alcohol yet is the less effective substrate for aldehyde synthesis. This observation indicates that differences in the chemistry of photosynthetic and H_2O_2-dependent O_2 evolution exist. One possible explanation for the increased effectiveness of glycerol as an inhibitor of H_2O_2-dependent O_2 evolution is that both hydroxides formed in step a of Figure 4 could be displaced by this inhibitor. Such a conformation would not result in the formation of aldehyde or O_2 (from H_2O_2) but would effectively inhibit both reactions. Further experiments are required to test this hypothesis.

The multiline EPR signal is believed to arise from a mixed-valence dimer or tetramer of manganese that contains Mn(III) and Mn(IV) (Dismukes & Siderer, 1981; Hansson et al., 1982; Brudvig et al., 1986). The origin of the $g = 4.1$ EPR signal is also believed to arise from a mixed-valence Mn(III)–Mn(IV) cluster (Brudvig et al., 1986) or from an almost axial Mn(IV) species (Aasa et al., 1987). Hansson et al. (1987) have interpreted the relationship of the $g = 4.1$ and the multiline EPR signals to result from a redox equilibrium between a dimer and a monomer of manganese. These workers proposed that changes in the reduction potentials of the components upon addition of alcohols (Zimmerman & Rutherford, 1986) drive the redox equilibrium of the manganese in favor of the Mn(III)–Mn(IV) dimer. This hypothesis is intriguing because water associated with the S_0–S_3 states during photosynthetic O_2 evolution is readily exchangeable with the bulk medium (Radmer & Ollinger, 1986), yet mononuclear Mn(IV) should be exchange inert (Cotton & Wilkinson, 1966). Since NH_3 promotes the $g = 4.1$ EPR signal at the expense of the multiline signal (Beck & Brudvig, 1986), inhibition of O_2 evolution by this reagent may result from the inability of catalytic ligands to exchange with the bulk medium.

Photosystem II preparations depleted of the 23- and 17-kDa proteins have been found to catalyze the formation of H_2O_2 when illuminated (Sayre & Homann, 1979; Schröder & Åkerlund, 1986). The reaction center complex preparations used in the present study also lack these extrinsic membrane proteins (Ghanotakis & Yocum, 1986). The production of H_2O_2 may result from a back-reaction of reaction a in Figure 4 after formation of the S_2 state in the light. Thus, a possible function of the 23- and/or 17-kDa protein may be to prevent this back-reaction.

The mechanism of H_2O_2-dependent aldehyde formation and O_2 evolution described above has been confined to the reactions

which involve the S_0 and S_2 states. However, the OEC can catalyze the formation of O_2 from H_2O_2 by a cycle that involves the S_1/S_{-1} states as well (Velthuys & Kok, 1978; Frasch & Mei, 1987a,b). If the reaction of H_2O_2 with the S_1 state has a mechanism similar to that described in Figure 4, then formation of the S_{-1} state must involve more than one of the four manganese in the cluster and probably involves a cycle between two Mn(III) and two Mn(II). A low molecular weight manganese dimer has been synthesized recently which will oxidize H_2O_2 to O_2 (Mathur et al., 1987). The catalase-like activity from this complex is thought to result from a cycle between two Mn(II) and two Mn(III).

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