The Oxygen-Evolving Complex Requires Chloride To Prevent Hydrogen Peroxide Formation†

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ABSTRACT: Illumination of PSII core preparations can cause the production of H_2O_2 at rates which approach 60 μmol of H_2O_2 (mg of Chl·h)$^{-1}$. The rate of peroxide production is maximal at pH 7.2 at low sucrose concentrations and at concentrations of Cl^- (1.5–3.0 mM) that limit the rate of the oxidation of water to O_2. The rate of H_2O_2 production increased with pH from pH 6.8 to 7.2 and was inversely proportional to the oxidation of water to O_2 from pH 6.8 to 7.5. While EDTA does not inhibit H_2O_2 production, this reaction is abolished by 5 mM NH_2OH and inhibited by the same concentrations of NH_3 that affect water oxidation which indicates that the oxygen-evolving complex is responsible for the production of peroxide generated upon illumination of PSII core preparations. These results support a mechanism in which bound Cl^- in the S_2 state is displaced by OH^- ions which are then oxidized by the OEC to form H_2O_2. Thus, the OEC requires Cl^- to prevent access to the active site of the OEC until four oxidizing equivalents can be generated to allow the oxidation of water to O_2.

The oxygen-evolving complex (OEC)[1] catalyzes the oxidation of water to molecular oxygen to supply electrons for the light reactions of photosystem II (PSII). Oxygen is evolved after the enzyme donates four electrons to the PSII reaction center in sequential one-electron reactions (Kok et al., 1971; Forbush et al., 1972). The five intermediate catalytic states formed by these sequential transitions are designated S_0–S_4, such that release of O_2 occurs upon formation of the S_4 state.

The OEC contains four bound manganese (Cheniae & Martin, 1971) and requires Cl^- (Hind et al., 1969; Kelley & Izawa, 1978) and Ca^{2+} (Miyao & Murata, 1984; Nakatani, 1984; Ghanotakis et al., 1984) for activity. Charge accumulation in the the S_0/S_1 and S_1/S_2 transitions has been shown to occur via manganese-centered oxidations by several independent methods (Dismukes & Siderer, 1981; Yachandra et al., 1986; Srinivasan & Sharp, 1986a,b; Styring & Rutherford, 1988). Chloride is required to form an S_2 state that is competent to allow subsequent S state transitions (Theg et al., 1984; Itoh et al., 1984). Removal of an extrinsic 23-kDa protein (OEE2) has been shown to inhibit O_2-evolving activity by decreasing the affinity of the OEC for Cl^- (Akerlund et al., 1982; Ikeuchi et al., 1985). However, increased levels of Cl^- can compensate for the effects caused by the loss of this protein. Restoration of activity to Cl^--depleted preparations can also be achieved with Br^-, NO_3^-, I^-, HCO_2^-, or HCO_3^- in order of decreasing effectiveness (Kelley & Izawa, 1978). The S_2 state is also specifically susceptible to inhibition by several unprotonated amines (Velthuys, 1975; Frasch & Cheniae, 1980). This inhibition has been found to result from the displacement of Cl^- from the OEC (Sandusky & Yocum, 1983, 1984) and, in most cases, causes the dissociation of OEE2 (Akerlund et al., 1982).

Alkaline treatments that can deplete the OEC of Cl^- without the loss of OEE2 (Kuwabara & Murata, 1979; Homann, 1985) are also known to inhibit O_2 evolution via a specific interaction with the S_2 state (Briantais et al., 1977). Using OEE2-depleted PSII preparations, Homann (1988) found that the rate of O_2-evolving activity decreased from its optimum at pH 6.5 with increasing pH at rate-limiting (V_{max}/K_M) concentrations of Cl^-. However, the V_{max} rate observed at pH 6.5 could be obtained at pH values as high as pH 7.4 in the presence of elevated Cl^- concentrations. From these observations, Homann (1988) hypothesized an ordered reaction in which a histidine residue with a $pK_a < 5$ becomes protonated and then binds Cl^-.

Using PSII core preparations Frasch and Mei (1987a,b) determined that the OEC can catalyze the conversion of H_2O_2 to O_2 in darkness by the interconversion of the S_0/S_2 and S_1/S_{-1} states. These reactions are insensitive to EDTA indicating that hexaquo Mn^{2+} is not responsible for the production of O_2 from peroxide. Mano et al. (1987) found that when the concentration of $H_2{}^{18}O_2$ exceeded 1 mM, PSII preparations evolved $^{18}O_2$ in lieu of $^{16}O_2$ from the oxidation of $H_2{}^{16}O$. The ability of the OEC to catalyze the oxidation of alcohols in the presence of H_2O_2 supports a reaction mechanism in which the enzyme first reduces peroxide in a two-electron reaction to form two bound hydroxides (Frasch et al., 1988). A second peroxide then displaces one of the hydroxides and reacts in a two-electron reaction to form O_2. This mechanism is supported by the observation that both oxygens in the product are derived from the same peroxide molecule (Mano et al., 1987).

Measurements of the flash yields of O_2 that originate from PSII show an anomalously high yield of O_2 on the first flash under a wide variety of conditions (Berg & Seibert, 1987). The high yield of O_2 observed after a single flash with inverted, salt-washed thylakoids was interpreted to result from the production of H_2O_2 by the preparation since the effect was

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCIP, 2,6-dichlorophenylindophenol; OEE1, extrinsic 33-kDa protein; OEE2, extrinsic 23-kDa protein; OEE3, extrinsic 17-kDa protein; MES, 2-morpholinoethanesulfonic acid; OEC, oxygen-evolving complex; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PPBQ, phenyl-*p*-benzoquinone; PSII, photosystem II; Tris, tris(hydroxymethyl)aminomethane.

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abolished by the addition of exogenous Fe-catalase (Schröder & Akerlund, 1986). Inhibition of the formation of this anomalous O_2 by EDTA suggested that the H_2O_2 produced after the flash was converted to O_2 by aqueous manganese. This was confirmed using $CaCl_2$-washed PSII preparations (Berg & Seibert, 1987). These latter experiments also showed that H_2O_2 was unable to release manganese from the OEC unless PSII was depleted of the 33-kDa protein (OEE1).

Schröder and Akerlund (1986) postulated that the H_2O_2 originates as an intermediate in the oxygen-evolving process, which is able to dissociate from the enzyme more easily with salt-treated thylakoids due to the absence of the OEE2 and 17-kDa (OEE3) proteins. Addition of concentrated sulfuric acid to PSII preparations also causes the release of peroxide which Klimov et al. (1990) believe to be the release of a bound intermediate of water oxidation. However, using $H_2{}^{18}O$ and mass spectroscopy, it has been shown that the O_2 evolved upon formation of the S_4 state is derived from water than can rapidly exchange from the solvent into the S_2 and S_3 states (Radmer & Ollinger, 1986). From this observation, it is difficult to explain the formation of bound peroxide in the S_2 or S_3 states as a catalytic intermediate in the normal mechanism of the OEC.

The conditions in which H_2O_2 was observed by anomalously high flash yields of O_2 on the first flash were carried out in the absence of an exogenous electron acceptor (Schröder & Akerlund, 1986). Subsequently, Schröder and Åkerlund (1990) used $H_2{}^{18}O$ and mass spectroscopy to show that, in the absence of an acceptor, H_2O_2 does not originate from the oxidation of water upon illumination of salt-washed preparations. This peroxide was postulated to originate instead from the reduction of O_2 via reactions on the reducing side of PSII, a reaction known to occur (Shuvalov & Krasnovsky, 1975).

Illumination of PSII preparations at low sucrose concentrations was also found to cause the production of H_2O_2 measured under steady-state conditions using a coupled assay with Fe-catalase (Wydrzynski et al., 1989). In these experiments, the ability to produce H_2O_2 was not changed when either DCIP, PPBQ, or PPBQ and ferricyanide were used to accept electrons. The rate of PSII electron transfer determined by DCIP reduction was also found to be greater than the rate measured as photosynthetic O_2 evolution. This difference largely disappeared when the rate of O_2 was measured in the presence of the exogenous Fe-catalase which converts the H_2O_2 produced to O_2. Under similar conditions, peroxide production was inhibited by NH_3 and inactivated by NH_2OH (Fine & Frasch, 1990). These results indicate that, in the presence of an electron acceptor, the fraction of electron transfer through PSII which was not expressed as the oxidation of water to O_2 was used for the production of H_2O_2 by the oxidizing side of PSII.

We now report studies of the mechanism in which H_2O_2 is generated as a result of illumination of PSII core complexes under steady-state conditions in the presence of an electron acceptor by examining the dependence of this reaction on the Ca^{2+}, Cl^-, and sucrose concentrations as well as pH. The use of core complexes was advantageous to this investigation because the octyl glucopyranoside used to purify the complexes removed most of the endogenous Fe-catalase which increases the ability to measure H_2O_2 production. This detergent also depletes the preparation of the OEE2 protein, Ca^{2+}, and Cl^- in a manner that is much less destructive to PSII activity than other treatments (Ghanotakis & Yocum, 1986). As a result, activity is recoverable by addition of Ca^{2+} and Cl^- in a greater fraction of reaction centers from PSII core preparations than can be obtained with salt-washed PSII preparations. The results presented here indicate that the OEC requires Cl^- in the S_2 state to prevent the enzyme from using the stored oxidizing potential to oxidize OH^- to H_2O_2.

Table I: Effect of EDTA and NH_2OH Treatments on H_2O_2 Production by PSII Core Preparations

treatment	rate[a] –catalase	rate[a] +catalase	% H_2O_2 production[b]
control	118	158	25
+100 μM EDTA	155	200	23
NH_2OH-washed[c]	0	0	

[a] Rates are given in units of micromoles of O_2 per milligram of chlorophyll per hour, as assayed using the conditions of Figure 1. [b] Calculated as the fraction of the difference between the rate with and without catalase divided by the rate with catalase. [c] Core preparations were incubated with 5 mM NH_2OH for 1 h, 4 °C in darkness, and then washed in 40 mM MES, pH 6.0, prior to assay.

MATERIALS AND METHODS

Photosystem II core preparations were isolated from spinach using the procedure of Ghanotakis and Yocum (1986). The core complexes were washed in a buffer of 40 mM MES–$(CH_3)_4NOH$, pH 6.0 which effectively removed Na^+, Ca^{2+}, and Cl^-. The preparations were then pelleted in an Eppendorf microfuge and resuspended to 100 μg of Chl/mL in the MES–$(CH_3)_4NOH$ buffer and stored at 77 K until needed.

Rates of O_2 evolution were measured using a Clark-type O_2 electrode at saturating light intensities with 400 μM 2,6-dichloro-*p*-benzoquinone (DCBQ) as an electron acceptor. The amplifier for the O_2 electrode was made by G. J. Johnson, Baltimore, MD, and is capable of measuring rates as low as 10 μmol of O_2 (mg of Chl·h)$^{-1}$ with precision. Measurements were routinely made in duplicate or triplicate within each experiment and any data point that varied by more than 5 μmol of O_2 (mg of Chl·h)$^{-1}$ was discarded. The experiment measuring the extent of hydroquinone inactivation as a function of the sucrose concentration was replicated five times. The number of replications for the other experiments is given in the figure legends.

To examine the effects of Cl^- at constant Ca^{2+}, Cl^- was added as $(CH_3)_4NCl$. Calcium was added from a stock solution of 50 mM $Ca(OH)_2$ in 200 mM MES or PIPES buffer adjusted to the desired pH with $(CH_3)_4NOH$. Hydrogen peroxide production was measured with a coupled assay using bovine Fe-catalase (Sigma). The amount of catalase required to observe maximal rates of H_2O_2 production was determined empirically before each experiment and was typically 0.25 mg of catalase/mL.

RESULTS

The rate of H_2O_2 production that resulted from illumination of PSII core preparations was measured by a coupled reaction with bovine Fe-catalase. Since this enzyme catalyzes the dismutation of H_2O_2 to O_2, the observed rate of O_2 evolution will reflect the combined rate of the oxidation of water to O_2 and H_2O_2 formation. As shown in Table I, the rate of O_2-evolving activity of PSII core complexes in the absence of Fe-catalase at 3 mM Cl^-, pH 7.0, was 75% of the rate measured in the presence of Fe-catalase. The effect of Fe-catalase on the rate was not decreased by EDTA which indicates that H_2O_2 production was not catalyzed by aqueous divalent cations. Instead, the generation of H_2O_2 appears to have been mediated by functional manganese of the OEC because the

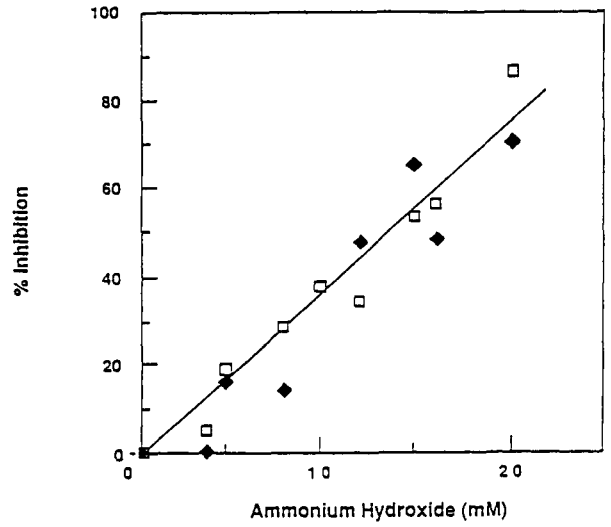

FIGURE 1: Inhibition by NH_3 of the rate of oxidation of water to O_2 (□) and of H_2O_2 production (◆) from PSII core preparations. The rate of H_2O_2 production was determined by the difference of the rate of O_2-evolving activity in the presence versus the absence of 0.5 mg of bovine Fe-catalase during the assay. The assay mixture contained 20 mM $Ca(OH)_2$–PIPES, 3.0 mM $(CH_3)_4NCl$, 400 μM DCBQ, 10 μg of Chl of PSII core preparations, and 80 mM PIPES, which was buffered to pH 7.0 with $(CH_3)_4NOH$. Ammonia was added as NH_4OH–PIPES, pH 7.0, at the concentrations indicated. The data shown are from one of eight replications.

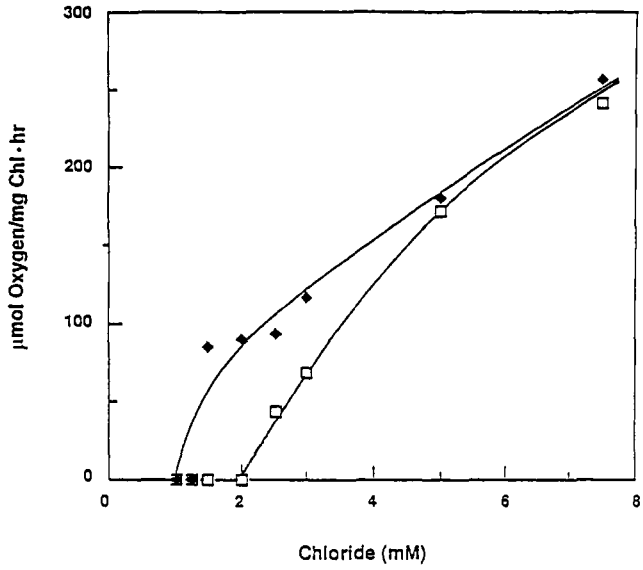

FIGURE 2: Chloride dependence of the rate of O_2-evolving activity catalyzed by illumination of PSII core preparations in the absence (□) and the presence (◆) of 0.5 mg of bovine Fe-catalase. Chloride was added to the reaction mixture as $(CH_3)_4NCl$. The reaction mixture also contained 20 mM $Ca(OH)_2$–PIPES, pH 7.0, 400 μM DCBQ, 10 μg Chl of PSII core preparations, and 80 mM PIPES–$(CH_3)_4NOH$, pH 7.0. The rate of H_2O_2 production was determined as in Figure 1. The maximal rate at this pH for the oxidation of water to O_2 of 482 μmol of O_2 (mg of Chl·h)$^{-1}$ was obtained at 20 mM Cl^-. The data shown represent one of three replicate experiments.

core preparations washed with 5 mM NH_2OH, a treatment known to remove functional manganese (Cheniae & Martin, 1971), abolished both the oxidation of water to O_2 and H_2O_2 production.

The involvement of the OEC in the generation of H_2O_2 is also indicated by the effect of NH_3 on the activity of PSII core preparations as shown in Figure 1. In this experiment, ammonia was added as NH_4OH buffered with PIPES, pH 7.0, such that the pH and Cl^- concentration remained constant. At 3 mM Cl^-, half-maximal inhibition of the oxidation of water to O_2 (squares) as well as H_2O_2 production (diamonds) was obtained in core preparations with unprotonated NH_3 concentrations of about 85 μM. The similarity in the dependence of both reactions on the NH_3 concentration suggests that both processes are catalyzed by the OEC. The unprotonated form of the amine, which has been established as the inhibitory species, induces half-maximal inhibition of water oxidation in thylakoids at concentrations of 40–900 μM (Velthuys, 1975; Frasch & Cheniae, 1980). This inhibition results, in part, from the displacement of Cl^- from the OEC (Sandusky & Yocum, 1983, 1984). The difference in sensitivity of thylakoids and PSII core preparations to this inhibitor may be explained by the lack of the OEE2 protein in the core preparations since depletion of OEE2 decreases the affinity for Cl^- (Akerlund et al., 1982; Ikeuchi et al., 1985; Ghanotakis & Yocum, 1986).

The dependence of the rate of H_2O_2 production on the concentration of Cl^- at pH 7.0 is shown in Figure 2. Hydrogen peroxide production only becomes measurable at concentrations of Cl^- which limit the rate of oxidation of water to O_2. The rate of H_2O_2 production was approximately equal to the rate of oxidation of water to O_2 at 2.5 mM Cl^- and accounted for all of the O_2-evolving activity at 2 mM Cl^-. However, at sufficiently low Cl^- concentrations, the Cl^- became rate limiting to H_2O_2 production as well.

Figure 3A shows the interdependence of the rate of H_2O_2 production (open symbols) and the rate of the oxidation of water to O_2 (closed symbols) as a function of pH at 3 mM Cl^-. The rate of water oxidation to O_2 catalyzed by the core preparations decreased over the pH range of 6.5 to 7.5 in a manner similar to that observed in NaCl-washed PSII preparations (Homann, 1988). At pH 6.5, which is optimal for the oxidation of water to O_2, H_2O_2 production was not observed. The rates of the oxidation of water to O_2 and H_2O_2 production were inversely proportional such that H_2O_2 production was favored under slightly alkaline conditions. Although at pH 7.5 all of the activity of these PSII core preparations resulted in H_2O_2 production, the maximal rate of H_2O_2 production was observed at pH 7.2 (Figure 3B).

The effect of Ca^{2+} on the fraction of PSII core particles that generate peroxide was examined in Figure 4. When the Cl^- and pH were held constant at 3.5 mM and 7.0, respectively, the rates of both the oxidation of water to O_2 and the production of peroxide increased by the addition of Ca^{2+} (Figure 4A). However, the magnitude of the increase in the rate of peroxide formation due to the addition of Ca^{2+}, as determined by the difference in the rate of O_2 evolved plus and minus Fe-catalase (closed squares), was small relative to the increase in the rate of O_2 production from water. As shown in Figure 4B, changing the Ca^{2+} concentration had little effect on the fraction of reaction centers producing peroxide upon illumination relative to the fraction that oxidized water to O_2. These results suggest that while Ca^{2+} increased the rate of light-driven electron flow in PSII, it did not convert PSII core preparations from a H_2O_2-forming mode to the oxidation of water to O_2.

Wydrzynski et al. (1989) observed that PSII preparations produced H_2O_2 in the presence of an electron acceptor only at low sucrose concentrations but not at low Cl^-. Such results are consistent with an explanation in which (i) low sucrose concentrations may cause the loss of OEE2, which reduces the affinity of the OEC for Cl^-, or (ii) sucrose may substitute for Cl^- binding to the OEC. To determine if low sucrose concentrations induce the dissociation of OEE2, PSII preparations prepared as per Berthold et al. (1981) were incubated with or without 400 mM sucrose, and then the rate of water

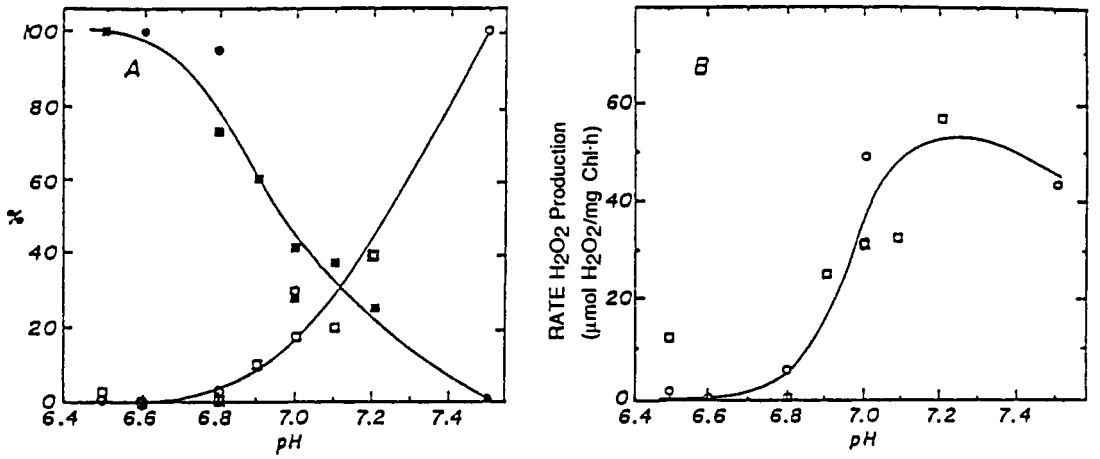

FIGURE 3: (A) Effect of pH on the relative fraction of the rate of the oxidation of water to O_2 (closed symbols) and H_2O_2 production (open symbols) catalyzed by the illumination of PSII core preparations at 3 mM Cl^- and 20 mM Ca^{2+}. Circles and squares represent separate experiments. Each point was replicated five times within each experiment. The reaction mixture contained 3 mM $(CH_3)_4NCl$, 20 mM $Ca(OH)_2$–PIPES, 500 μM DCBQ, and 10 μg of Chl of PSII core preparations. The pH of the reaction mixture was maintained with 40 mM MES and 40 mM PIPES buffered to the pH indicated with $(CH_3)_4NOH$. The fraction of H_2O_2 production was determined by the difference in the rate of O_2-evolving activity in the presence versus the absence of 0.5 mg of bovine Fe-catalase divided by the rate with catalase. The maximal rate at pH 6.5 for the oxidation of water to O_2 of 605 μmol of O_2 (mg of Chl·h)$^{-1}$ was obtained at 20 mM Cl^-. (B) The dependence of the rate of H_2O_2 production on pH. The rates were calculated from the data of panel A expressed as micromoles of O_2 per milligram of Chl per hour.

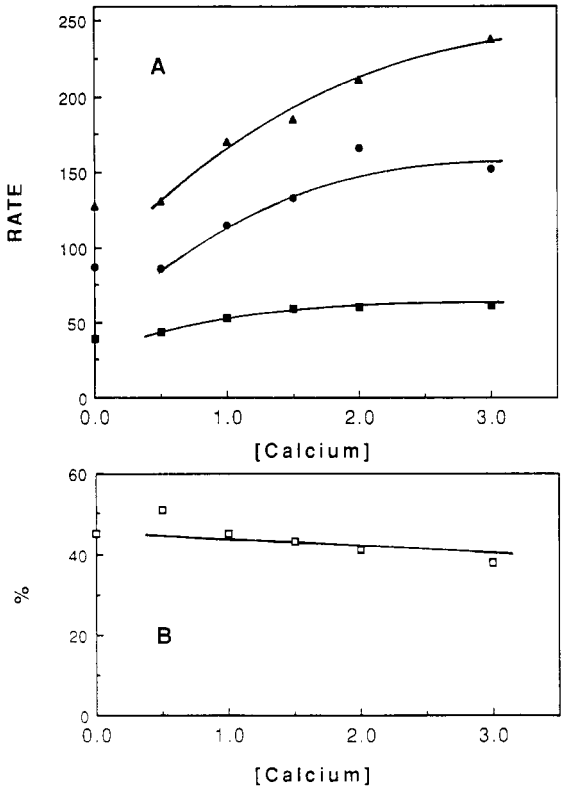

FIGURE 4: (A) Calcium dependence of the rate of O_2-evolving activity catalyzed by illumination of PSII core preparations in the absence (●) and the presence (▲) of 0.5 mg of bovine Fe-catalase. In addition to the concentration of $Ca(OH)_2$–PIPES, pH 7.0, indicated, the assay mixture contained 3.5 mM $(CH_3)_4NCl$, 400 μm DCBQ, 10 μg of Chl of PSII core preparations, and 80 mM PIPES which was buffered to pH 7.0 with $(CH_3)_4NOH$. Regression analysis of the double-reciprocal plots of the rates of O_2 evolution was used to obtain the best fit to the data. The rate of H_2O_2 production (■) was determined by the difference of the best fits in the presence versus the absence of Fe catalase. (B) The fraction of H_2O_2 production was determined as the rate without catalase divided by the rate with catalase. The data shown are from one of four replicate experiments.

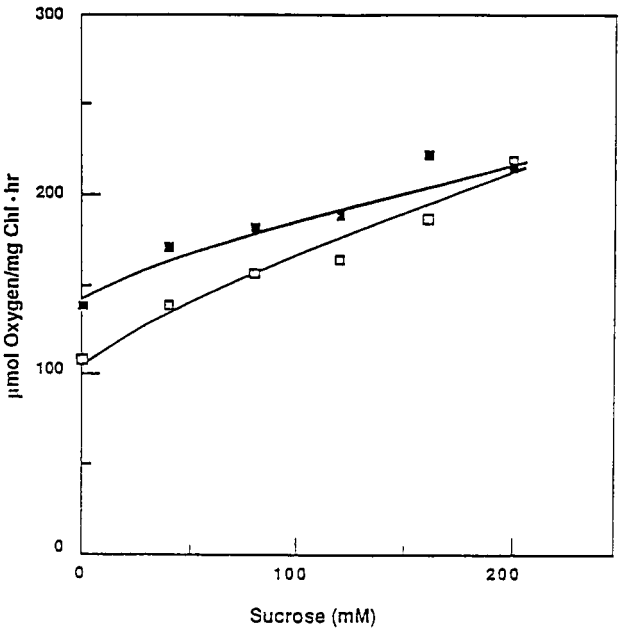

FIGURE 5: Sucrose dependence of the rate of O_2-evolving activity catalyzed by illumination of PSII core preparations in the absence (□) and the presence (■) of 0.5 mg of bovine Fe-catalase. In addition to the concentrations of sucrose indicated, the assay mixture contained 20 mM $Ca(OH)_2$–PIPES, pH 7.0, 3.0 mM $(CH_3)_4NCl$, 400 μM DCBQ, 10 μg of Chl of PSII core preparations, and 80 mM PIPES–$(CH_3)_4NOH$, pH 7.0. The data shown are the average of measurements made in triplicate and represent one of three replicate experiments.

oxidation to O_2 was examined for changes in the Ca^{2+} requirement and for the extent of hydroquinone inactivation known to occur in OEE2-depleted PSII preparations (Ghanotakis et al., 1984). No changes in the Ca^{2+} requirement or sensitivity to hydroquinone were observed in PSII preparations that had incubated in the absence of sucrose for as long as 6 h (data not shown) which indicates that sucrose does not alter the affinity of the OEC for Cl^- by causing the dissociation of OEE2.

The effect of sucrose on the activity of PSII core preparations in 3 mM Cl^-, pH 7.0, is shown in Figure 5. Illumination of the core preparations at this pH and Cl^- concentration in the absence of sucrose shows an elevated rate of O_2 evolution in the presence of Fe-catalase indicative of peroxide formation. As the concentration of sucrose increased, the rate at which

water was oxidized to O_2 increased while the proportion of the activity due to H_2O_2 production decreased. These results suggest that sucrose may partially substitute for Cl^- via a direct interaction with the OEC. However, the effectiveness of sucrose in preventing the generation of peroxide is small relative to that of chloride.

DISCUSSION

The OEC Can Catalyze H_2O_2 Production via the S_2 State. The involvement of the functional manganese of the OEC in the production of H_2O_2 by PSII core preparations is indicated by the lack of inhibition by EDTA and the inhibition or inactivation by the same concentrations of NH_3 and NH_2-OH, respectively, that affect the oxidation of water to O_2. Furthermore, the effects of Cl^-, OH^-, and NH_3 on H_2O_2 production presented here show that peroxide is produced upon formation of the S_2 state. The experiments presented here were assayed with 400 μM DCBQ. The presence of an electron acceptor eliminates the acceptor side of PSII as a source of H_2O_2 production from results that showed (i) that the addition of ferricyanide to keep the electron acceptor PPBQ oxidized did not affect the rate of H_2O_2 production and (ii) that the rate of H_2O_2 production accounted for the difference between the rate of DCIP reduction and photosynthetic O_2 evolution (Wydrzynski et al., 1989).

The H_2O_2 produced on the oxidizing side of PSII is shown here to result from depletion of Cl^- from the oxygen-evolving complex by the inverse relationship between Cl^- and H_2O_2 formation and by the effects of OH^- and NH_3 on the rate of peroxide production. We have also confirmed the observation (Wydrzynski et al., 1989) that H_2O_2 production increases at low sucrose concentrations. Sucrose has been documented to affect the binding of Cl^- to the OEC (Beck & Brudvig, 1988) and, thus, the effect of sucrose on peroxide formation is probably due to an alteration on the binding of Cl^- or to the ability of sucrose to block access of other molecules to the Cl^- site.

Despite our ability to demonstrate that sucrose can prevent the formation of peroxide, our results still conflict with those of Wydrzynski et al. (1989), who claimed that Cl^- depletion was not involved with H_2O_2 formation. This apparent contradiction is probably the result of the methodology used to deplete the system of Cl^-. Wydrzynski et al. (1989), cautious of side effects that might be introduced by Cl^--depletion methods such as high pH shock or addition of chelators or high concentrations of other anions (Ghanotakis & Yocum, 1986; Homan, 1987) that might lead to peroxide formation unrelated to the Cl^- binding site, removed the anion from PSII preparations simply by washing these preparations in 20 mM MES, pH 6.3. This protocol is not very effective in depleting PSII preparations of Cl^- since these preparations contain the extrinsic membrane proteins that facilitate the binding of the anion with high affinity. However, subsequent washes in high sucrose and low Cl^- probably allowed sucrose to displace functional Cl^- via mass action such that subsequent low sucrose treatments gave rise to the formation of peroxide in a manner similar to that presented here using PSII core preparations. The use of core preparations was advantageous because they have been clearly shown to be depleted of Ca^{2+}, Cl^-, and the extrinsic membrane proteins, yet are highly active when supplemented with these ions (Ghanotakis & Yocum, 1986).

The Substrate of H_2O_2 Production Is OH^-. The evidence presented here supports the mechanism of H_2O_2 production shown in Figure 6. In this mechanism, light-driven PSII

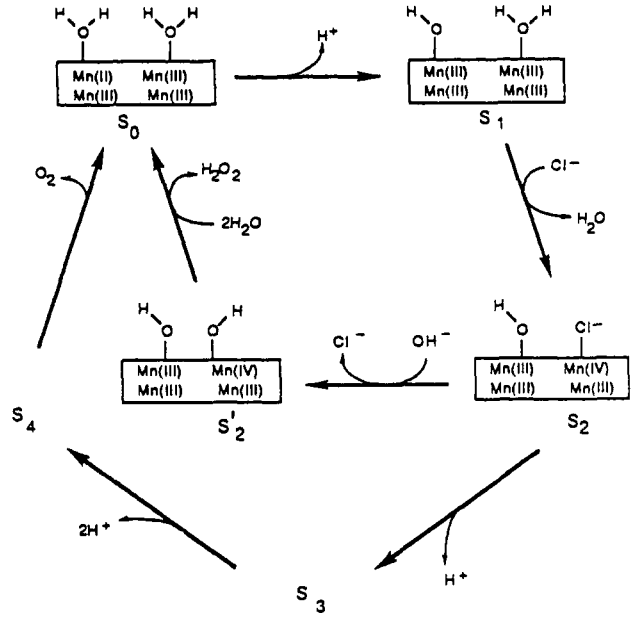

FIGURE 6: Proposed mechanism of H_2O_2 production by the OEC upon illumination of PSII core preparations. The exchangeable coordination of ligands to the manganese cluster shown is not intended to specify a particular Mn.

reactions drive the normal S_0/S_1 and S_1/S_2 transitions of the OEC, which involve the oxidation of manganese (Yachandra et al., 1986; Srinivasan & Sharp, 1986a,b; Styring & Rutherford, 1988). PSII reaction centers contain a tightly bound Cl^- (Lindberg et al., 1990) and recent EXAFS analyses suggest that about one Cl^- is coordinated to the manganese cluster in the S_2 state (Klein et al., 1991). While there is evidence that water molecules can coordinate to the manganese in the S_2 state (Hansson et al., 1986), mass spectroscopy experiments using $H_2{}^{18}O$ indicate that this water must be in rapid exchange with the bulk solvent (Radmer & Ollinger, 1986).

Oxidation of the manganese in the S_0/S_1 and S_1/S_2 transitions will make the metal a stronger Lewis acid which will cause the coordinated water to become a correspondingly stronger Lewis base. As a result, water coordinated to manganese in the S_1 state and to a greater extent in the S_2 state probably exists as OH^-. Formation of OH^- as a result of the S_0/S_1 transition is consistent with the observation that this step involves the release of a proton (Saphon & Crofts; 1977; Junge et al., 1977). However, formation of an S_2 state capable of making an S_2/S_3 transition does not result in the release of a proton (Saphon & Crofts; 1977; Junge et al., 1977) but instead requires Cl^- (Theg et al., 1984; Itoh et al., 1984). From the interdependence between Cl^- and OH^- that gives rise to the inverse relationship for the formation of H_2O_2 or the oxidation of water to O_2 shown here, it is clear that the oxidation of water is diverted to H_2O_2 production when the concentration of OH^- is sufficient to displace Cl^-. Inhibition of the oxidation of water to O_2 by OH^- is known to result from a specific interaction with the S_2 state (Briantais et al., 1977) and is consistent with the competitive inhibition kinetics by OH^- versus Cl^- of the oxidation of water to O_2 (Homann, 1988; Critchley et al., 1982).

By examining unstacked thylakoids at pH 7.8, Jahns et al. (1991) reported that H^+ were released from the S state transitions beginning with S_0/S_1 with a stoichiometry of 1:0.5:1:1.5. This noninteger pattern of proton release at pH 7.8 is consistent with the model in Figure 6 since the model predicts that, at this pH, water would displace Cl^- in a significant

fraction of the reaction centers. This would result in the release of a H^+ in the fraction of reaction centers that contain water during the S_1/S_2 state transition. The proton release pattern as a function of pH (Rappaport & Lavergne, 1991) shows that the S_1/S_2 state transition does not involve the release of a H^+ at pH 6.0 and that the increased yield of H^+ follows the pH dependence for peroxide formation shown in Figure 3. Protonatable amino acid side chains may mediate proton release from the OEC, but evidence for the existence of these side chains is currently lacking and, thus, such details have been omitted from the mechanism in Figure 6.

We propose that if two OH^- can become bound to the S_2 state of the OEC, the enzyme can catalyze a two-electron oxidation reaction that produces H_2O_2 and results in the formation of the S_0 state. We have previously found that addition of high concentrations of H_2O_2 drives this reaction in reverse (Frasch & Mei, 1987a,b). The peroxide-induced formation of the dihydroxide-bound S_2 state (S_2') from S_0 was shown by the observation that the S_2 state formed from S_0 by H_2O_2 in the dark was capable of oxidizing primary alcohols to aldehydes (Frasch et al., 1988). Production of this S_2' state by peroxide is also supported by the observation that the O_2 produced by the OEC from a mixture of $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$ is composed of either $^{16}O^{16}O$ or $^{18}O^{18}O$ but not $^{16}O^{18}O$ (Mano et al., 1987) (i.e., that both oxygens in the product are derived from the same peroxide molecule). It is of note that the four-electron oxidation of 4 OH^- to form O_2 and H_2O only requires a potential of 0.40 V which would make this reaction highly favorable upon formation of the S_4 state. The suitability of OH^- as the substrate for the oxidation of water to O_2 is also favored by Krishtalik (1990) on the basis of theoretical analysis of activation energies.

Chloride May Prevent Oxidation of Substrate in Lower S States. Since the enzyme oxidizes water in a four-electron reaction but must accumulate each oxidizing equivalent successively, the results presented here suggest that Cl^- prevents the OEC from oxidizing the substrate until all four equivalents (the S_4 state) have been obtained. If this is correct, then the lower S states have a redox potential less than that required to oxidize Cl^- ($E^{\circ\prime}$ = 1.36 V). While a number of reagents have been shown to substitute for Cl^- in the OEC, the relative effectiveness of these substitutes must be limited by their redox potentials. For example, although I^- ($E^{\circ\prime}$ = 0.54 V) can substitute for Cl^- (Kelley & Izawa, 1978), I^- can donate electrons to PSII (Ikeuchi et al., 1988). Since the redox potential for the oxidation of water to peroxide at pH 7 ($E^{\circ\prime}$ = 1.35 V) is comparable to that of Cl^-, the OEC should not be capable of generating H_2O_2 from water. However, the potential for the oxidation of OH^- to peroxide ($E^{\circ\prime}$ = 0.93 V) is much more favorable. The close correlation between the pH dependence for H_2O_2 production in Figure 3 and the inhibition of O_2-evolving activity observed by Homann (1985) provides further support that the substrate for H_2O_2 production is OH^-.

The conditions described here that lead to the production of peroxide would, in the presence of catalase, be predicted to give rise to a pattern for the flash-induced yields of oxygen in which the yield is maximal on the first flash and every second flash thereafter. At this time, the only report of flash yields of oxygen under conditions that promote peroxide formation were carried out using salt-washed, inverted thylakoids (Schröder & Akerlund, 1986) which showed the predicted pattern for the first four flashes. Unfortunately, these experiments were performed in the absence of an electron acceptor, and the bulk of the peroxide was later shown to arise from reactions on the reducing side of PSII (Schröder & Akerlund, 1990).

The formation of bound peroxide as an intermediate oxidation product in the S_2 or S_3 states has been proposed (Renger, 1987; Matsushita et al., 1988; Klimov et al., 1990) and it is possible that the peroxide we have observed here results from the dissociation of this peroxidic intermediate from the enzyme when Cl^- is limiting. However, this hypothesis is unlikely because dissociation of a putative peroxidic intermediate would require protonation and, thus, would be expected to dissociate faster with decreasing pH. This conflicts with the pH dependence for peroxide formation shown in Figure 3. Krishtalik (1990) has shown from calculations of activation energies that a favorable mechanism for the production of O_2 by the OEC would be via a rate-determining oxidation of OH^- and H_2O to H_2O_2 followed by the rapid oxidation of peroxide to O_2. There is currently no evidence to preclude these reactions from occurring upon formation of the S_4 state.

Peroxide can react with proteins and is known to damage cellular components (Tew & Ortiz de Montellano, 1988). We have recently shown that when peroxide is produced by the OEC upon illumination, it can react with the PSII reaction center and contribute to the process known as photoinhibition (Bradley et al., 1991). The presence of Fe-catalase in the stacked regions of the thylakoids (Nakatani & Barber, 1981; Frasch & Mei, 1987) probably serves to minimize this type of photoinhibition by scavenging H_2O_2 produced by the OEC.

The Cl^- Binding Site May Be the Site of Water Oxidation in the OEC. The fact that H_2O_2 production occurs upon displacement of the functional Cl^- bound to the OEC implicates this Cl^- binding site as the site of water oxidation. The site of water oxidation was first postulated to be the site of inhibition for the unprotonated form of NH_3 (Velthuys, 1975) and Tris (Frasch & Cheniae, 1980) on the basis of the similarity of unprotonated amines with water. While bulky amines like Tris were found to compete for the binding of Cl^-, the mixed noncompetitive inhibition kinetics of NH_3 versus Cl^- suggested that NH_3 also inhibited O_2-evolving activity at a second site that did not involve Cl^- (Sandusky & Yocum, 1983, 1984; Homann, 1986). Beck and Brudvig (1986) concluded that this second site was the water-oxidizing site from experiments describing the effects of NH_3 on the EPR signals that arise from the OEC–manganese. However, subsequent experiments showing that O_2-evolving activity is not blocked when NH_3 is bound only to the non-Cl^- site and that NH_3 must bind to a second site to inhibit the enzyme led Boussac et al. (1990) to conclude that the Cl^- binding site is the catalytic site of O_2 production. These latter findings concur with the results presented here in terms of the interactions between Cl^- and NH_3 and the production of peroxide.

The data of Figure 3 also show that at sufficiently low Cl^- concentrations the ability of the OEC to oxidize water to form either peroxide or O_2 is blocked, indicating that Cl^- must have an additional role in limiting light-induced electron flow. Depletion of Cl^- by high pH excursion or by the use of PSII core particals results in an increase in the g = 4.1 EPR signal that arises from the S_2 state manganese at the expense of the multiline EPR signal (Casey & Sauer, 1984; Ono et al., 1986). When the Cl^- bound to PSII preparations was displaced by F^- (Baumgarten et al., 1990), illumination caused changes in the S_2 state EPR signals normally associated with Cl^--depletion but also induced the 160 g EPR signal and decreased the intensity of EPR signal II. These latter effects were previously associated with the depletion of Ca^{2+} (Boussac et al., 1990).

We have found that while Ca^{2+} increases the rate of electron flow in PSII reaction centers that produce both O_2 and H_2O_2, this cation did not alter the proportion of reaction centers that catalyzed peroxide formation. When combined with the observation that F^- is unable to inhibit peroxide formation (G. C. Dismukes, personal communication), it appears that Cl^- may also act at a site not involved with H_2O_2 formation that facilitates electron transfer from the manganese cluster to Y_Z^+. Further work is required to address these questions.

REFERENCES

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

Baumgarten, M., Philo, J., & Dismukes, G. (1990) *Biochemistry 29*, 10814–10822.

Beck, W., & Brudvig, G. (1986) *Biochemistry 25*, 6479–6486.

Beck, W., & Brudvig, G. (1988) *Chem. Scr. 28A*, 93–98.

Berg, S., & Seibert, M. (1987) *Photosynth. Res. 13*, 3.

Bertold, D., Babcock, G., & Yocum, C. (1981) *FEBS Lett. 61*, 231–234.

Boussac, A., Rutherford, A. W., & Styring, S. (1990) *Biochemistry 29*, 24–32.

Bradley, R. L., Long, K. M., & Frasch, W. D. (1991) *FEBS Lett. 286*, 209–213.

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

Casey, J. L., & Sauer, K. (1984) *Biochim. Biophys. Acta 767*, 21–28.

Cheniae, G. M., & Martin, I. R. (1971) *Biochim. Biophys. Acta 253*, 167–181.

Dismukes, G. C., & Siderer, Y. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 274–278.

Fine, P., & Frasch, W. D. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I. 3, pp 905–908, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Forbush, B., Kok, B., & McGloin, M. (1972) *Photochem. Photobiol. 11*, 257–275.

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

Frasch, W. D., & Mei, R. (1987a) *Biochim. Biophys. Acta 891*, 8–14.

Frasch, W. D., & Mei, R. (1987b) *Biochemistry 26*, 7321–7325.

Frasch, W. D., & Mei, R., & Sanders, M. (1988) *Biochemistry 27*, 3715–3719.

Ghanotakis, D., & Yocum, C. (1986) *FEBS Lett. 197*, 244–248.

Ghanotakis, D., Topper, J., & Yocum, C. (1984) *Biochim. Biophys. Acta 767*, 524–531.

Hind, G., Nakatani, H., & Izawa, Y. (1969) *Biochim. Biophys. Acta 180*, 388–398.

Homann, P. (1985) *Biochim. Biophys. Acta 809*, 311–319.

Homann, P. (1986) *Photosynth. Res. 10*, 497–502.

Homann, P. (1987) *J. Bioenerg. Biomembr. 19*, 105–123.

Homann, P. (1988) *Biochim. Biophys. Acta 934*, 1–13.

Ikeuchi, M., Yuassa, M., & Inoue, Y. (1985) *FEBS Lett. 179*, 60–64.

Ikeuchi, M., Koike, H., & Inoue, Y. (1988) *Biochim. Biophys. Acta 932*, 160.

Itoh, S., Yerkes, C., Koike, H., Robinson, H., & Crofts, A. (1984) *Biochim. Biophys. Acta 766*, 612–622.

Jahns, P., Lavergne, J., Rappaport, F., & Junge, W. (1991) *Biochim. Biophys. Acta 1057*, 313–319.

Kelley, P. M., & Izawa, S. (1978) *Biochim. Biophys. Acta 502*, 189–210.

Klein, M., Yachandra, V., DeRose, V., Mukerji, I., Latimer, M., & Sauer, K. (1992) *Proceedings of the International Conference on Bioinorganic Chemistry*, VCH Publishers, New York (in press).

Klimov, V., Ananyev, G., Allakhverdiev, S., Zharmukhamedov, S., Mulay, M., Hedge, U., & Padhye, S. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I.3, pp 247–254, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kok, B., Forbush, B., & McGloin, M. (1971) *Photochem. Photobiol. 14*, 307–321.

Krishtalik, L. (1990) *Bioelectrochem. Bioenerg. 23*, 249–263.

Kuwabara, T., & Murata, N. (1979) *Biochim. Biophys. Acta 581*, 228–236.

Lindberg, K., Wydrzynski, T., Vänngård, T., & Andréasson, L.-E. (1991) *FEBS Lett. 264*, 153–155.

Mano, J., Takahashi, M., & Asada, K. (1987) *Biochemistry 26*, 2495–2501.

Matsushita, T., Spencer, L., & Sawyer, D. (1988) *Inorg. Chem. Lett. 27*, 1167–1173.

Miyao, M., & Murata, N. (1984) *FEBS Lett. 168*, 118–120.

Nakatani, H. (1984) *Biochem. Biophys. Res. Commun. 120*, 299–304.

Ono, T., Zimmermann, J., Inoue, Y., & Rutherford, A. (1986) *Biochim. Biophys. Acta 851*, 193–201.

Quensel, J., & Akerlund, H.-E. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) Vol. I.3, pp 897–900, Kluwer, Academic Publishers, Dordrecht, The Netherlands.

Radmer, R. (1979) *Biochim. Biophys. Acta 546*, 418.

Radmer, R., & Ollinger, O. (1986) *FEBS Lett. 195*, 285.

Rappaport, F., & Lavergne, J. (1991) *Biochemistry 30*, 10004–10012.

Renger, G. (1987) *Photosynthetica 21*, 203–224.

Sandusky, P., & Yocum, C. (1983) *FEBS Lett. 162*, 339–343.

Sandusky, P., & Yocum, C. (1984) *Biochim. Biophys. Acta 766*, 603–611.

Schröder, W., & Akerlund, H.-E. (1986) *Biochim. Biophys. Acta 848*, 359.

Shuvalov, V., & Krasnovsky, A. (1975) *Biochimia (USSR) 40*, 358–367.

Srinivasan, A., & Sharp, R. (1986a) *Biochim. Biophys. Acta 850*, 211–217.

Srinivasan, A., & Sharp, R. (1986b) *Biochim. Biophys. Acta 851*, 369–376.

Styring, S., & Rutherford, A. W. (1988) *Biochemistry 27*, 4915–4923.

Tew, D., & Ortiz de Montellano, P. (1988) *J. Biol. Chem. 263*, 17880–17886.

Theg, S., Jursinic, P., & Homann, P. (1984) *Biochim. Biophys. Acta 766*, 636–646.

Velthuys, B. (1975) *Biochim. Biophys. Acta 396*, 392–401.

Wydrzynski, T., Angström, J., & Vännegård, T. (1989) *Biochim. Biophys. Acta 973*, 23–28.

Yachandra, V., Guiles, R., McDermott, A., Britt, R., Cole, J., Dexheimer, S., Sauer, K., & Klein, M. (1986) *J. Phys. C8*, 1121–1128.

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