Catalase-Free Photosystem II: The O_2-Evolving Complex Does Not Dismutate Hydrogen Peroxide†

Yelena G. Sheptovitsky and Gary W. Brudvig*

Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107

Received November 25, 1997; Revised Manuscript Received February 19, 1998

ABSTRACT: A photosystem II (PSII) membrane-associated heme catalase has been identified as a major source of the dark H_2O_2-dismutation reaction in PSII membrane samples [Sheptovitsky, Y. G., and Brudvig, G. W. (1996) *Biochemistry 35,* 16255−16263]. Based on this finding, a catalase-free PSII membrane sample was prepared by using mild heat treatment to deplete most of the PSII membrane-associated heme catalase followed by inhibition of the residual catalase with 50 mM 3-amino-1,2,4-triazole, a specific heme catalase inhibitor that binds covalently to compound I. After these treatments, the PSII membrane sample exhibited only 0.02% of the original H_2O_2-dismutation activity when assayed in the presence of 20 mM 3-amino-1,2,4-triazole. This small residual H_2O_2-dismutation activity is attributed to adventitious metal ions or the non-heme iron in PSII because the activity was still present in a Mn-depleted PSII sample but was completely suppressed by adding 5 mM ferricyanide to the assay buffer; the effect of ferricyanide is attributed to oxidation of H_2O_2-dismutating cations. Although the H_2O_2-dismutation activity was completely eliminated by these treatments, the light-induced O_2-evolution activity was retained. A single saturating flash given to catalase-free PSII membranes did not induce any H_2O_2-dismutation activity. These results demonstrate that the S_1/S_{-1} and S_2/S_0 cycles of the O_2-evolving complex of PSII do not occur in the presence of H_2O_2, as proposed by Velthuys, B., and Kok, B. [(1978) *Biochim. Biophys. Acta 502,* 211−221]. The light-induced O_2-evolution activity in catalase-free PSII was found to be irreversibly impaired by micromolar concentrations of H_2O_2. Thus, it is possible that the PSII membrane-associated heme catalase plays an important role in protection of the O_2-evolving complex from damage by H_2O_2.

Photosystem II (PSII)[1] is a thylakoid membrane protein complex responsible for light-driven O_2 evolution in plants. This is a highly energetic process, and it involves a number of redox species located in the D1 and D2 polypeptides of PSII (reviewed in ref *1*). A molecule of O_2 is produced from two water molecules in four light-driven electron-transfer steps. These electron-transfer steps begin with the photoexcitation of a special chlorophyll called P680 followed by the primary charge separation between the electron donor and acceptor sides of PSII. The O_2-evolving complex (OEC), a tetramanganese cluster on the donor side of PSII, oxidizes water while four electrons are consecutively transferred to the acceptor side of PSII in order to reduce plastoquinones bound at the Q_B site. In the process of water oxidation, the OEC cycles between five redox states, S_0 to S_4. The structure of the OEC, as well as the molecular mechanism of water oxidation, is yet to be determined. Because hydrogen peroxide is a potential intermediate in the evolution of O_2 from water, there has been much interest in the chemistry of hydrogen peroxide with the OEC, as it might provide insights on the mechanism of O_2 evolution from water.

The reaction of hydrogen peroxide with flash-illuminated chloroplasts was studied by Velthuys and Kok (*2*). A two-step delay in the flash-induced O_2-yield pattern was observed in chloroplast samples pretreated in the dark with 80 mM H_2O_2 at pH 8.8 for 1 min. These data demonstrated the reduction of the S_1 state to the S_{-1} state by hydrogen peroxide. Since chloroplasts did not show a significant conversion into the S_{-1} state after preincubation with H_2O_2 at neutral pH, Velthuys and Kok (*2*) concluded that an H_2O_2-dismutation cycle between the S_1 and S_{-1} states of the OEC takes place in the dark at neutral pH. When chloroplasts were given a single flash to produce the S_2 state in the presence of 8 mM H_2O_2 at pH 7.8 (conditions when formation of the S_{-1} state was minimal), most of the S_2 state was found to be converted into the S_0 state. Also, S_{-1}-enriched chloroplasts evolved O_2 after a single flash. Based on these results, a flash-induced H_2O_2-dismutation activity of the OEC was also proposed involving an S_2/S_0 cycle.

H_2O_2-dismutation reactions of the OEC were studied later by other groups. Frasch and Mei (*3, 4*) reported that the S_2/S_0 H_2O_2-dismutation rate is maximal when the chloride concentration is above 10 mM, and the dark H_2O_2-dismutation reaction of the OEC requires activation with Ca(II). It was reported that PSII core preparations catalyze the S_1/S_{-1} reaction in the presence of 130 mM H_2O_2 at rates up to 760 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (*5*). The rate of hydrogen

† This work was supported by the National Institutes of Health (GM32715).

[1] Abbreviations: AT, 3-amino-1,2,4-triazole; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCBQ, 2,5-dichloro-*p*-benzoquinone; DPC, *sym*-diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving complex; PPBQ, *p*-phenylbenzoquinone; PSII, photosystem II; PSII-30 °C, pellet fraction following incubation of PSII membranes at 30 °C for 90 min; PSII-30 °C-AT, PSII-30 °C treated with 50 mM AT and 10 mM ascorbate; snt-30 °C, supernatant fraction following incubation of PSII membranes at 30 °C for 90 min.

S0006-2960(97)02872-9 CCC: $15.00
Published on Web 03/26/1998

peroxide dismutation was found to be at least 3−10 times faster for the flash-induced S_2/S_0 cycle (*2, 6*−*9*).

Mn-depleted PSII samples were also found to evolve O_2 from H_2O_2 either after a single flash or under continuous light (*2, 10*). Two mechanisms appear to be involved. Some oxidation of H_2O_2 was mediated by metal ions because 0.5 mM EDTA decreased O_2 evolution from hydrogen peroxide by 23%. One-electron oxidation of H_2O_2, forming superoxide, was concluded to be responsible for the remaining portion of the evolved O_2 because 77% of the O_2 yield was inhibited with 1 mM ascorbate, a compound that reacts with superoxide, and this inhibition was partially reversed by superoxide dismutase. The latter mechanism of O_2 evolution from H_2O_2 could be similar to the mechanism of N_2 production from hydroxylamine in Mn-depleted PSII (*11, 12*). It is interesting to note that N_2 evolution from NH_2OH first was detected in water-oxidizing PSII after a single flash (*13*). Later, Beck and Brudvig (*11*) found that nitroxide radicals of *N*,*N*-dimethylhydroxylamine are only formed in Mn-depleted PSII membranes. The data suggested that the flash-induced N_2 evolution from hydroxylamine is the result of a one-electron reaction catalyzed by the PSII centers which had lost manganese during the treatment with NH_2OH. The light-induced formation of N_2 from hydroxylamine and superoxide from H_2O_2 probably both result from a one-electron reaction with the tyrosine Z radical, $Y_Z^{\bullet}$, based on studies of the reduction of $Y_Z^{\bullet}$ by exogenous electron donors in Mn-depleted PSII centers (*14, 15*).

There have been a number of reports that hydrogen peroxide can be produced by PSII during illumination (*6, 7, 16*−*22*). Using a luminol−peroxidase assay for the detection of H_2O_2, Klimov et al. (*19*) observed four types of flash-induced H_2O_2 signals, called signals A, B, C, and D, from PSII membrane preparations. Signal A was found to be due to the reduction of O_2 by the electron-acceptor side of PSII, as was previously reported by Schröder and Åkerlund (*23*). Signals B, C, and D were correlated with the electron-donor side of PSII based on the effects of flash illumination, although the source of hydrogen peroxide was ambiguous because these signals were generated only slowly after an untreated or treated sample was added to the luminol−peroxidase assay medium (signals B and D, respectively) or after treatment with acid (signal C). Thus, signals B, C, and D were assigned to "bound" H_2O_2. There are two possible explanations for the production of hydrogen peroxide by the OEC. First, "bound" H_2O_2 could be an intermediary product of water oxidation, which would be oxidized further to O_2 under normal conditions. Alternatively, it is possible that hydrogen peroxide is formed from a side reaction of water with high-valent manganese in the oxidized OEC upon addition of the sample to the luminol−peroxidase assay medium or during the acid treatment (*19*). In this regard, the treatments which promote signal D (lauroylcholine chloride, salt washing, or Cl^--depletion), as well as the high pH of the luminol−peroxidase assay (pH 8.0), perturb the donor side of PSII and may promote increased accessibility of the manganese cluster to H_2O/OH^- molecules with subsequent formation of H_2O_2 (*24*). Therefore, at the present time, it is not clear whether hydrogen peroxide is a product of a side reaction or an intermediate of the normal molecular pathway of water oxidation, although the concept of a side reaction has been favored (*7, 25, 26*).

Studies of the H_2O_2-production and dismutation reactions of the OEC have been complicated by a high-background H_2O_2-dismutation reaction in PSII. Most of this activity was attributed to the presence of a PSII membrane-associated heme catalase (*8, 27*). Although 1 mM NaN_3 or KCN has been used in H_2O_2-dismutation assays to inhibit the heme catalase, there was always a question of whether the residual H_2O_2-dismutation activity was coming from either the noninhibited portion of the heme catalase or the OEC. Another complication of adding azide/cyanide is that these reagents also inhibit PSII (*27*). Surprisingly, in the studies of the photoproduction of hydrogen peroxide discussed above, the samples were not treated with azide or cyanide to suppress the high background catalase activity. Therefore, it is possible that hydrogen peroxide production, if any, could not be detected because it was simply dismutated by the PSII membrane-associated catalase. In the case of H_2O_2 production from PSII samples treated to expose the donor side, the various treatments also might have resulted in the removal/inactivation of the PSII membrane-associated catalase which, thereby, could have facilitated the detection of hydrogen peroxide produced upon illumination (*28*).

Hydrogen peroxide can also damage PSII. Sandusky and Yocum (*29*) reported that the manganese cluster was released from chloride-depleted PSII membranes after treatment with high concentrations of hydrogen peroxide. The free manganese ions formed in this way could catalyze H_2O_2 disproportionation or oxidation chemistry in PSII samples, thus complicating the interpretation of H_2O_2-dismutation reactions of the OEC. Miyao et al. (*30*) also showed that the D1 protein is damaged at a site near the non-heme iron in various PSII samples treated with 10 mM H_2O_2. This protein damage was not observed in isolated reaction centers depleted of the non-heme iron. Iron(II) in a biological coordination environment is known to react with hydrogen peroxide much faster than hexaaqua-iron(II) in Fenton chemistry (*31*).

Recently, we reported the isolation and characterization of the PSII membrane-associated heme catalase from PSII membranes (*28*). In this work, we present a procedure for preparing catalase-free PSII membranes. This has allowed us to examine the dark- and flash-induced H_2O_2-dismutation activities of the OEC and the photoproduction of hydrogen peroxide in PSII samples in which the background H_2O_2-dismutation activity was eliminated.

MATERIALS AND METHODS

Sample Preparation. PSII membranes were isolated from market spinach leaves as described in Beck et al. (*32*) by a modified version of the procedure of Berthold et al. (*33*). All preparations were done on ice under dim green light. Prior to use, PSII membranes were stored at 3−5 mg of Chl/mL in liquid nitrogen. The resuspension buffer for PSII samples contained 15 mM NaCl, 20 mM MES, pH 6.5, and 30% ethylene glycol. The chlorophyll concentration was assayed by the method of Arnon (*34*). A stoichiometry of 200 Chl per PSII was used for calculations of PSII molar concentrations (*35*). The manganese cluster was removed from PSII samples by treatment with hydroxylamine according to Tamura and Cheniae (*36*). PSII membranes were depleted of the extrinsic polypeptides by 1 M $CaCl_2$

treatment; the O_2-evolution activity of $CaCl_2$-treated PSII membranes was typically about 60% of the activity of untreated PSII membranes when assayed in resuspension buffer containing 200 mM NaCl and 20 mM $CaCl_2$, in agreement with past work (*37*).

Heat-treated PSII membranes (PSII-30 °C), which are depleted of the PSII membrane-associated catalase, were prepared according to Sheptovitsky and Brudvig (*28*). PSII membranes (1.5−2.0 mg of Chl/mL) were incubated in a thermostated cell for 90 min with stirring at 30 °C followed by two cycles of centrifugation and resuspension of the pellet in resuspension buffer. Catalase-inhibited PSII membranes (PSII-30 °C-AT) were prepared by inhibiting catalase as described in Margoliash et al. (*38*) with minor modifications. PSII-30 °C (0.3 mg Chl/mL) were incubated with 50 mM 3-amino-1,2,4-triazole (AT), a specific heme catalase inhibitor which binds covalently to compound I, and 10 mM ascorbate for 2 h at 30 °C; ascorbate was used as a source of trace amounts of hydrogen peroxide to produce compound I. After incubation, the pellet was washed 2 times in resuspension buffer. A PSII-30 °C-AT preparation containing 20 mM AT and 5 mM ferricyanide in the buffer is denoted as catalase-free PSII membranes.

Assays. O_2-evolution measurements were made with either a YSI Model 53 O_2 monitor or an Instech Model 203B oxygen uptake system, each equipped with a Teflon-membrane-covered Clark-type O_2 electrode. Both sample cells were thermostated at 25 °C with a Neslab RTE-9DD circulator bath. The electrodes were calibrated with air-saturated water for which the O_2 concentration was taken as 0.25 mM. The O_2-assay buffer contained 20 mM MES, 15 mM NaCl, 20 mM $CaCl_2$, pH 6.5. For continuous-light-induced O_2-evolution activity assays, 5 mM ferricyanide and 0.5 mM DCBQ were added as electron acceptors, and the sample was illuminated via a tapered light pipe with saturating light from a 1000-W quartz-halogen lamp filtered by a heat-absorbing (Schott KG-5) and long-pass filter (Oriel LP 610). For dark H_2O_2-dismutation activity assays, the reaction rate was found to be proportional to both the PSII and H_2O_2 concentrations; the concentrations of PSII and H_2O_2 were chosen to obtain reproducible measurements, depending on the H_2O_2-dismutation activity.

Production of H_2O_2 in illuminated PSII membrane samples was assayed with a YSI Model 53 O_2 monitor as follows. In the case of PSII-30 °C-AT, a 250 μg Chl sample was added to 2.5 mL of the O_2-assay buffer containing 8 mM ferricyanide, 2 mM DCBQ, and 10 mM AT. In the case of 1 M $CaCl_2$-treated PSII-30 °C-AT, a 50 μg Chl sample was added to 2.5 mL of assay buffer containing 200 mM NaCl, 1 mM EDTA, 80 μg/mL bovine serum albumin, 2 mM ferricyanide, 1 mM PPBQ, and 20 mM MES, pH 6.3. The samples were then illuminated for 5 min in the assay chamber under continuous saturating light. Following illumination, O_2 production from H_2O_2 was monitored after addition of 5 μL of 0.1 mg/mL bovine liver catalase (Sigma) to the assay medium. At the end of each experiment, an aliquot of H_2O_2 was added to the assay medium to verify that the catalase was active and to calibrate the O_2 yield.

Flash experiments were performed with a Xe-flash tube (EG&G) with an 8 μF capacitor which produced an energy output of 4 J per flash at 1000 V. The flash-induced O_2-evolution measurements were made with the Instech micro-cell Clark-type electrode in order to avoid reaction of H_2O_2 at the electrode, as is the case with a bare platinum electrode, while still allowing single-flash measurements. However, owing to the slow time response of a Clark-type electrode, O_2-evolution measurements could not be made after each flash in a flash series. Therefore, flash-induced O_2-evolution measurements were made after a specific number of flashes spaced 1 s apart. The flashes were saturating for the concentrations of PSII membranes used.

For measurements of the dark- and flash-induced H_2O_2-dismutation activities of PSII-30 °C-AT preparations, a 125 μg Chl sample in O_2-assay buffer was placed into the 600 μL Instech assay chamber. 20 mM AT, 0.2 mM H_2O_2, and 5 mM ferricyanide were added, and the sample was incubated for 10−15 min to allow full inhibition of the residual PSII membrane-associated heme catalase and H_2O_2 dismutation by metal ions. Then 1 mM H_2O_2 was added and, after stabilization of the base line (1−2 min), O_2 evolution was monitored in the dark for 3−4 min. Then a single flash was given to the sample, and the flash-induced O_2 evolution was recorded. As soon as the experiment was completed, a 10 μg Chl aliquot was taken for an O_2-evolution assay under continuous light.

Electron-transfer activities of Mn-depleted PSII samples were measured spectrophotometrically using DPC and DCIP as the exogenous electron donor and acceptor, respectively (*39*). A 8 μg Chl sample was added to 1 mL of assay buffer containing 25 mM MES−NaOH, pH 6.5, 10 mM NaCl, 20 mM AT, 30 μM DCIP, 0.5 mM DPC, and, where indicated, 0.1 mM DCMU. The sample was continuously illuminated via a fiber optic with light from a 200 W Oriel lamp filtered by a heat-absorbing (Schott KG-5) and a 550 nm short-pass (Oriel) filter. Photoreduction of DCIP was monitored by the decrease of absorbance at 600 nm using a Perkin-Elmer Lambda 3B spectrophotometer. Absorption data were digitized with a strip-chart program written in Labview (National Instrument Corp.) on a Macintosh SE computer.

EPR measurements were performed at liquid helium temperatures on a Varian E-line EPR spectrometer equipped with an Oxford Instruments ESR 900 liquid helium cryostat. The recording conditions were the following: microwave power, 0.2 mW; temperature, 6.0 K; magnetic field modulation amplitude, 20 G; magnetic field modulation frequency, 100 kHz; microwave frequency, 9.285 GHz.

RESULTS

Preparation of Catalase-Free Photosystem II Membranes. Past studies of the H_2O_2-dismutation activity of the OEC have been done in the presence of azide or cyanide to inhibit the background heme catalase activity. However, 1 mM NaN_3 did not fully inhibit the H_2O_2-dismutation activity and also inhibited the light-induced O_2-evolution activity by about 30% (Table 1). Therefore, we have developed a procedure to prepare catalase-free PSII membranes without the use of azide or cyanide. This procedure involved three steps. First, most of the PSII membrane-associated heme catalase was depleted by a mild heat treatment (*28*). Second, the residual catalase was irreversibly inhibited by treatment with AT. Third, 20 mM AT and 5 mM ferricyanide were added to the sample before assay of the activity. The rates of light-induced O_2 evolution and disproportionation of H_2O_2 by PSII membranes after these treatments are presented in Table 1.

Table 1: Light-Induced O_2-Evolution and Dark H_2O_2-Dismutation Activities of Photosystem II Membrane Samples

sample	O_2-evolution rate[a,b] (%)	H_2O_2-dismutation rate[b,c] (%)
(a) untreated PSII membranes	100 ± 2	100 ± 1
(b) PSII-30 °C	89 ± 4	35 ± 3
(c) PSII-30 °C-AT	79 ± 8	1.7 ± 0.2
(d) Mn-depleted PSII-30 °C-AT	−	3.9 ± 0.3
(e) (a) + 1 mM NaN_3[d]	72	0.1
(f) (c) + 20 mM AT[d]	76	0.02
(g) (f) + 5 mM ferricyanide[d]	64	<0.005

[a] 100% = 580 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. [b] Standard deviations were determined by repeating the series of treatments on the same preparation of PSII membranes. [c] 100% = 890 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (mM H_2O_2)$^{-1}$. [d] After additions, the samples were incubated at 0.2 mg of Chl/mL for 5 min and then 10 μg Chl aliquots were taken for O_2 assays.

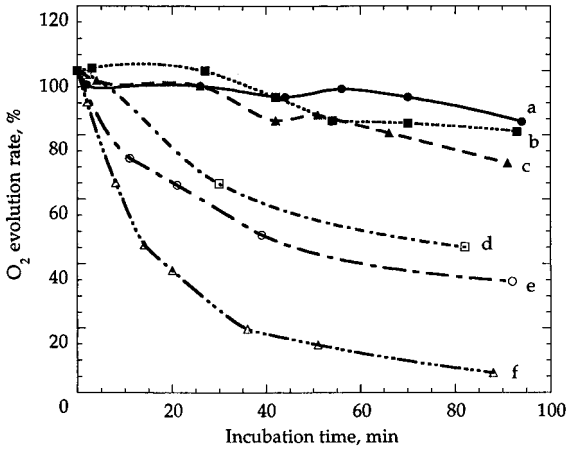

FIGURE 1: Effect of incubation time on the O_2-evolution activity of PSII membranes. PSII-30 °C-AT (0.2 mg of Chl/mL) were incubated at 25 °C in 20 mM MES, 15 mM NaCl, and 20 mM $CaCl_2$ at pH 6.5 after the following additions were made: (a) no additions; (b) 20 mM AT; (c) 5 mM ferricyanide; (d) as in (b) plus 0.2 mM H_2O_2; (e) as in (b) plus 5 mM ferricyanide and 0.2 mM H_2O_2; (f) as in (b) plus 5 mM ferricyanide and 1.2 mM H_2O_2. 10 μg Chl aliquots of the sample were taken for O_2-evolution assays after various incubation times. The lines drawn though each data set were interpolated for clarity.

Mild heat treatment of PSII membranes decreased the H_2O_2-dismutation activity by more than 60%. The H_2O_2-dismutation activity was further reduced by treatment with 50 mM AT and 10 mM ascorbate to less than 2% of the initial activity, or 0.02% if 20 mM AT was present in the assay buffer. The remaining 0.02% of the activity was eliminated upon addition of 5 mM ferricyanide to the assay buffer. Although the H_2O_2-dismutation activity was completely inhibited by these treatments, most of the light-induced O_2-evolution activity was retained. Depletion of the manganese cluster from PSII-30 °C-AT did not eliminate the residual H_2O_2-dismutation activity. Just the opposite, the residual activity was somewhat higher in the Mn-depleted sample. The small increase of the H_2O_2-dismutation activity of Mn-depleted PSII-30 °C-AT compared to PSII-30 °C-AT is attributed to removal of free AT from the sample during the washing steps used for Mn depletion.

Inhibition of the OEC by Hydrogen Peroxide. Figure 1 shows the effects of AT, ferricyanide, and hydrogen peroxide on the light-induced O_2-evolution activity in PSII-30 °C-AT. Inhibiting the H_2O_2-dismutation activity by adding either 20 mM AT or 5 mM ferricyanide did not change the light-induced O_2-evolution activity of PSII-30 °C-AT, as curves b and c coincide with curve a for the PSII-30 °C-AT sample which had no additions during incubation. The light-induced O_2-evolution activity of catalase-free PSII membranes was quite sensitive to hydrogen peroxide. 0.2 mM H_2O_2 caused significant inactivation of O_2 evolution of PSII-30 °C-AT when 20 mM AT was present (curve d), and the inhibition was more pronounced when 5 mM ferricyanide was also added (curve e). 1.2 mM H_2O_2 caused an even greater inactivation of O_2 evolution (curve f).

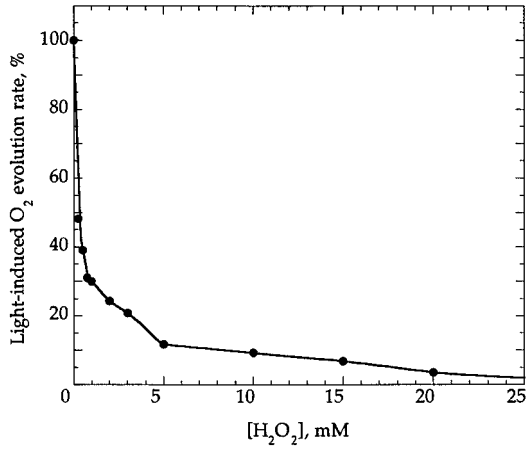

FIGURE 2: Effect of hydrogen peroxide on the O_2-evolution activity of PSII membranes. PSII-30 °C-AT (0.2 mg of Chl/mL), 20 mM AT, and 5 mM ferricyanide were incubated at 25 °C for 20 min with various concentrations of hydrogen peroxide.

The inhibitory effect of hydrogen peroxide on the light-induced O_2-evolution activity of catalase-free PSII was studied (Figure 2). Incubation for 20 min with 0.25 mM H_2O_2 resulted in inactivation of over 50% of the light-induced O_2-evolution activity, and about 90% inactivation occurred when the H_2O_2 concentration was 5 mM. The inhibition of O_2-evolution activity by H_2O_2 was not reversed by adding bovine liver catalase to decompose the H_2O_2 remaining after the incubation. One possibility is that H_2O_2 could cause the release of Mn(II) ions from PSII, as was found when Cl^--depleted PSII membranes were treated with H_2O_2 (*29*). However, the EPR spectrum of catalase-free PSII which had been incubated with 120 mM H_2O_2 for 20 min showed only a slight increase in the six-line Mn(II) and rhombic Fe(III) EPR signals; the EPR-visible Mn(II) accounted for less than 10% of the PSII centers (*40*).

Photoproduction of Hydrogen Peroxide by Catalase-Free Photosystem II. Our preparation of catalase-free PSII provides a system to test for the photoproduction of hydrogen peroxide by PSII. Both catalase-free PSII and 1 M $CaCl_2$-treated catalase-free PSII were studied. To inhibit the residual H_2O_2-dismutation activity in PSII-30 °C-AT, 10 mM AT and 8 mM ferricyanide were added to the assay media. Following illumination of the sample for 5 min, bovine liver catalase was injected into the assay medium. No O_2 evolution from H_2O_2 was detected. Based on the detection limit of our system (0.1 μM H_2O_2) and the concentration of PSII used in the assay (0.1 mg of Chl/mL, approximately 0.5 μM PSII), we conclude that the net amount of H_2O_2 evolved at the end of the illumination was less than 0.2 per PSII. The same result was obtained for 1 M $CaCl_2$-treated PSII-30 °C-AT. It should be mentioned that, in the latter case, the

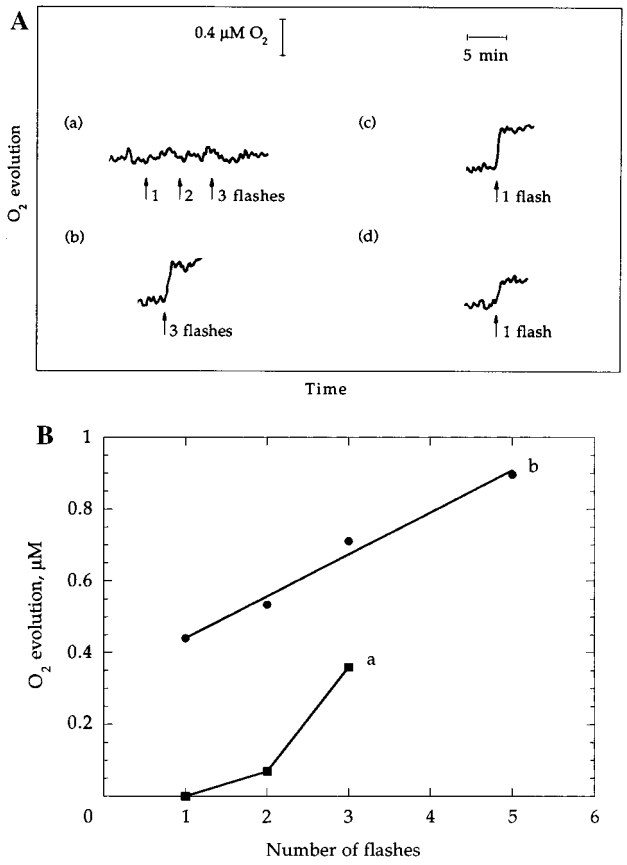

FIGURE 3: (A) O_2-evolution traces from (a) assay buffer in the presence of 20 mM AT, 5 mM ferricyanide, and 3 mM H_2O_2 after one, two, and three flashes; (b) 125 μg of Chl of PSII-30 °C-AT after 3 flashes in the presence of 1 mM ferricyanide and 0.25 mM DCBQ; (c) 125 μg of Chl of Mn-depleted PSII-30 °C-AT in the presence of 20 mM AT, 5 mM ferricyanide, and 3 mM H_2O_2 after a single flash; (d) 125 μg of Chl of PSII-30 °C-AT in the presence of 20 mM AT, 5 mM ferricyanide, and 3 mM H_2O_2 after a single flash. The arrows denote the times of the flashes. (B) Dependence of the O_2-evolution yields on the number of flashes given to (a) 125 μg of Chl of PSII-30 °C-AT and (b) 125 μg of Chl of Mn-depleted PSII-30 °C-AT in the presence of 20 mM AT, 5 mM ferricyanide, and 3 mM H_2O_2.

addition of AT was not necessary as these samples did not exhibit any dark H_2O_2-dismutation activity.

Flash-Induced H_2O_2 Dismutation by Catalase-Free Photosystem II. The results of flash experiments with a catalase-free PSII membrane preparation are reported in Figure 3. Figure 3Aa shows that one, two or three flashes did not induce any signal in the control sample containing only assay buffer with 20 mM AT, 5 mM ferricyanide, and 3 mM H_2O_2. Figure 3Ab,Ba shows that PSII-30 °C-AT did not produce significant amounts of O_2 after one or two flashes, and after three flashes O_2 evolution was observed at the saturation level. The O_2 yield after three flashes was 0.43 ± 0.05 μM O_2 (0.46 O_2 molecule per 200 Chl, average of six measurements). Figure 3Ac shows the result from a single flash given to a sample of Mn-depleted catalase-free PSII containing 3 mM H_2O_2. The flash induced an immediate O_2 production of 0.44 ± 0.05 μM O_2 (0.48 O_2 molecule per 200 Chl, average of five measurements). Figure 3Bb shows that the yield of O_2 from Mn-depleted catalase-free PSII containing 3 mM H_2O_2 increased linearly with flash number, although the average yield per flash was less than the first-flash yield. Figure 3Ad shows the result from a single flash given to a sample of catalase-free PSII containing 3 mM H_2O_2. The O_2 yield in this case was 0.26 ± 0.05 μM O_2 (0.28 O_2 molecule per 200 Chl, average of 13 measurements), which was about 60% of the first-flash O_2 yield from a Mn-depleted catalase-free PSII sample. The flat pre-flash base line in Figure 3Ad demonstrates that the sample did not exhibit any measurable dark H_2O_2-dismutation activity. The O_2 production in catalase-free PSII occurred only immediately following the flash; continuous O_2 evolution, as would be expected in the case of a S_2/S_0 H_2O_2-dismutation cycle of the OEC, was not observed. O_2-evolution activity assays of the catalase-free PSII sample were done after the flash experiment and showed that the sample retained about 40% of the light-induced O_2-evolution activity owing to some inactivation by the addition of 3 mM H_2O_2.

Light-Dependent O_2 Evolution from H_2O_2 in Mn-Depleted Catalase-Free Photosystem II. The origin of the O_2 evolved from H_2O_2 in Mn-depleted catalase-free PSII following a single flash (Figure 3Ac,Bb) was further investigated. The flash-induced production of O_2 from H_2O_2 in Mn-depleted catalase-free PSII was linearly dependent on the number of flashes spaced 1 s apart. With continuous illumination, Mn-depleted catalase-free PSII evolved O_2 from a 3 mM solution of H_2O_2 at a rate of 12 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. This concentration of hydrogen peroxide appeared to be saturating, as the same rate was observed from 6 mM H_2O_2. DCBQ in the assay media was necessary in order to obtain maximal rates and yields of O_2 evolution. To verify that the light-induced production of O_2 from H_2O_2 derived from PSII electron transport, measurements were made in the presence of 0.1 mM DCMU; 0.1 mM DCMU decreased both the rate and the yield of O_2 evolution from H_2O_2 by 44%. The incomplete inhibition by DCMU was found to be due to an effect of hydrogen peroxide on the binding of DCMU to PSII, rather than to a non-PSII mechanism for the light-induced production of O_2 from H_2O_2; 0.1 mM DCMU completely blocked electron transport from DPC to DCIP in Mn-depleted PSII-30 °C-AT. After the sample was pretreated with 3 mM H_2O_2, however, 0.1 mM DCMU inhibited DPC to DCIP electron transport by only 45%, although the pretreatment with hydrogen peroxide did not significantly affect the rate of electron transport from DPC to DCIP in the absence of DCMU. Therefore, we conclude that hydrogen peroxide affects the ability of DCMU to bind to the Q_B site without significantly disrupting electron transport. From these results, we can also conclude that all of the light-induced production of O_2 from H_2O_2 in Mn-depleted PSII-30 °C-AT derives from PSII electron transport.

DISCUSSION

We have developed a procedure for preparing PSII membrane samples free of background H_2O_2-dismutation activity from the PSII membrane-associated heme catalase and metal ions. These catalase-free PSII samples retained most of the light-induced O_2-evolution activity, but they were irreversibly inactivated by micromolar concentrations of hydrogen peroxide. Using catalase-free PSII membranes and conditions when inactivation of O_2-evolution activity by hydrogen peroxide was minimal, we have examined the dark- and flash-induced H_2O_2-dismutation activities of the S_1/S_{-1} and S_2/S_0 cycles of the OEC. The catalase-free PSII samples

have also enabled us to study the photoproduction of H_2O_2 by PSII membranes.

Dark H_2O_2-Dismutation Reaction of the O_2-Evolving Complex. We have found that there are two sources of dark H_2O_2-dismutation activity in PSII membranes. A PSII membrane-associated heme catalase is the major source and is responsible for all but 0.02% of the activity, as shown by the effects of depleting the heme catalase by mild heat treatment and of adding the heme catalase-specific inhibitor, 3-amino-1,2,4-triazole (Table 1). The small residual H_2O_2-dismutation activity was still present in a Mn-depleted PSII sample but was completely suppressed by adding 5 mM ferricyanide to the assay buffer. The effect of ferricyanide can be explained by oxidation of adventitious metal ions or the non-heme iron. Metal ions such as Cu(II), Mn(II), and Fe(II) are known to catalyze H_2O_2 dismutation. Fe(II), in particular, also reacts with hydrogen peroxide via Fenton chemistry (*31*). Therefore, we conclude that adventitious Fe(II), Cu(II), or Mn(II) or the non-heme iron in PSII were the second source of the dark H_2O_2-dismutation activity in PSII membranes.

Many research groups have used azide or cyanide to inhibit the PSII membrane-associated heme catalase in order to study the dark- and flash-induced H_2O_2-dismutation reactions of the OEC in PSII samples (*2*−*4*, *8*−*10*). However, as seen in Table 1, the PSII membrane-associated catalase is not completely inhibited by 1 mM NaN_3; cyanide is less inhibitory than azide (*28*). Therefore, azide- or cyanide-treated PSII samples would exhibit some residual H_2O_2-dismutation activity. In addition, the H_2O_2-dismutation activity of adventitious metal ions in PSII samples would not be eliminated by adding azide or cyanide. Consequently, there has always been a background rate of H_2O_2 dismutation in past studies of the dark- and flash-induced H_2O_2-dismutation reactions of the OEC. By suppressing both sources of the dark H_2O_2-dismutation activity in PSII membranes while maintaining good light-induced O_2-evolution activity, we can conclude that the S_1/S_{-1} H_2O_2-dismutation cycle of the OEC does not occur at a measurable rate. It should be noted that this result does not raise doubt about the reduction of the S_1 state to the S_{-1} state by H_2O_2. Measurements of a two-flash delay in the O_2 flash-yield pattern of H_2O_2-treated chloroplasts provide convincing evidence that this reaction took place and was favored by high pH (*2*). Moreover, the observation that high concentrations of H_2O_2 cause the release of Mn(II) ions from Cl^--depleted PSII samples provides additional evidence that H_2O_2 acts as a reductant of the manganese ions in the OEC (*29*). However, the conclusion by Velthuys and Kok (*2*) that the OEC could be reoxidized by hydrogen peroxide, thus completing a dismutation cycle, was only inferred from the pH dependence of hydrogen peroxide dismutation and not based on a direct measurement of the reaction.

Light-Induced H_2O_2-Dismutation Reaction of the O_2-Evolving Complex. The light-induced H_2O_2-dismutation rate from the S_2/S_0 cycle has been estimated to be 3−10 times higher than the dark rate from the S_1/S_{-1} cycle (*2*, *6*, *8*, *9*, *27*). We can use the previously reported rates of dark- and light-induced H_2O_2 dismutation from PSII preparations to estimate the expected rate of H_2O_2 dismutation from the S_2/S_0 cycle under our conditions. The rate of the dark S_1/S_{-1} H_2O_2-dismutation cycle in PSII core preparations was measured to be 760 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} in the presence of 130 mM H_2O_2 (*5*). This rate can be recalculated for a PSII membrane preparation assayed at low concentrations of H_2O_2, if the following two results are used. The chlorophyll contents in PSII membrane and core PSII preparations are about 200 and 50 molecules/PSII center, respectively (*27*, *35*), and the rate of the H_2O_2-dismutation reaction is proportional to the H_2O_2 concentration. Using these values and the literature H_2O_2-dismutation rates, a PSII membrane preparation would be expected to have a rate of the dark H_2O_2-dismutation reaction of about 1.5 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (mM H_2O_2)$^{-1}$ and a rate of the S_2/S_0 H_2O_2-dismutation reaction in the range of 4−15 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} (mM H_2O_2)$^{-1}$. We found that a single flash given to a catalase-free PSII membrane sample produced only a small fraction of the O_2 expected for the S_2/S_0 H_2O_2-dismutation reaction and the O_2 was generated immediately after the flash. We did not observe catalytic O_2 evolution from H_2O_2 after a single saturating flash with a rate that decayed slowly according to the lifetime of the S_2 state, as has been reported for the S_2/S_0 H_2O_2-dismutation reaction (*10*). Therefore, we conclude that there is no significant S_2/S_0 H_2O_2-dismutation activity in catalase-free PSII membranes. The yield of O_2 generated immediately after a flash in catalase-free PSII was about 60% of the yield measured in Mn-depleted catalase-free PSII. Since catalase-free PSII also lost approximately 60% of the water-oxidizing activity during the flash experiment due to inactivation by H_2O_2, we conclude that the one-flash-induced evolution of O_2 that we observe from catalase-free PSII membranes derived from H_2O_2 oxidation in damaged centers.

H_2O_2 Photooxidation by Mn-Depleted Catalase-Free Photosystem II. Flash- or light-induced O_2 evolution from H_2O_2 in Mn-depleted PSII has been reported previously (*2*, *8*). We determined the O_2 yield in Mn-depleted catalase-free PSII to be 0.48 O_2 molecule per 200 Chl when the concentration of H_2O_2 was saturating, which is consistent with the yield observed by Mano et al. (*8*), taking into consideration that the contribution of metal ions to H_2O_2 dismutation was eliminated in our PSII samples. Mano et al. (*8*) also provided evidence that light-induced evolution of O_2 from H_2O_2 by Mn-depleted PSII proceeds via formation of superoxide. Based on this observation and our measured flash yield of approximately 0.5 O_2/PSII, we propose the following two-step reaction mechanism for the production of O_2 from H_2O_2 by Mn-depleted PSII:

$$H_2O_2 + Y_Z^{\bullet} \rightarrow O_2^- + H^+ + Y_Z \quad (1)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

These reactions would only occur in Mn-depleted or damaged PSII centers, where $Y_Z^{\bullet}$ becomes accessible to hydrogen peroxide.

Formation of Hydrogen Peroxide by Photosystem II. Previous studies have shown that H_2O_2 could be formed by PSII in free and "bound" forms (*19*). However, these studies have been performed in the presence of the PSII membrane-associated heme catalase, which could have dismutated any photoproduced H_2O_2. In samples of either catalase-free PSII or 1 M $CaCl_2$-treated catalase-free PSII samples, we found that the net yield of photoproduced hydrogen peroxide was

less than 0.2 per PSII after 5 min of illumination. These results demonstrate that PSII does not photoaccumulate significant amounts of free hydrogen peroxide, although we would not have observed "bound" H_2O_2 under our assay conditions. This result is consistent with our observation that the light-induced O_2-evolution activity of PSII samples did not decrease in parallel with inhibition of the H_2O_2-dismutation activity (Table 1); if a significant amount of H_2O_2 was being generated in the light, then the O_2-evolution activity of catalase-free PSII would have been much smaller than that of untreated PSII.

There are, however, mechanisms for the light-induced oxidation of H_2O_2 by either Mn-depleted PSII (eqs 1 and 2) or Mn-containing PSII (eqs 3 and 4).

$$H_2O_2 + S_1 \rightarrow O_2 + S_{-1} \quad (3)$$

$$S_{-1} + 2h\nu \rightarrow S_1 \quad (4)$$

In both cases, light is used to effect the oxidation of H_2O_2 to O_2. Therefore, it is not possible to exclude the photoproduction of some hydrogen peroxide by PSII which was decomposed during the illumination via either eqs 1 and 2 or eqs 3 and 4.

Sensitivity of Catalase-Free Photosystem II to H_2O_2. Micromolar concentrations of hydrogen peroxide significantly inactivated the light-induced O_2-evolution activity in catalase-free PSII samples (Figure 2). The inactivation was more significant for samples in which the dark H_2O_2-dismutation reaction was more strongly inhibited (Figure 1, curves d and e). Hydrogen peroxide has been shown to reduce the OEC to the S_{-1} state (*2*), and, in the case of Cl^--depleted PSII, the reduction of the OEC is accompanied with the release of Mn(II) (*29*). We found that H_2O_2 did not significantly inactivate PSII electron transport and also did not cause the release of Mn(II) in Cl^--sufficient PSII. Therefore, the irreversible inactivation of O_2-evolution activity by H_2O_2 appears to be caused by disruption of the manganese cluster without release of Mn(II) ions. The mechanism of H_2O_2 inhibition of the donor side of PSII might be similar to the one of irreversible hydroxylamine inhibition of the OEC (*11*), except that in the case of H_2O_2 the conditions are more oxidizing such that free Mn(II) ions are not formed.

In all previous studies of the H_2O_2-dismutation activity of the OEC, high concentrations of H_2O_2 (up to 300 mM) were used (*2*−*4*, *8*, *10*). Without inhibiting the background heme catalase activity, much of the added H_2O_2 may have been dismutated, resulting in a lower and time-dependent concentration of H_2O_2. However, our data suggest that most of the O_2-evolving centers should be damaged with such high concentrations of H_2O_2, especially under conditions when the PSII membrane-associated catalase was inhibited. Therefore, measurements of light-induced O_2 evolution from H_2O_2 in PSII samples must take into account the degree of PSII inactivation because Mn-depleted PSII samples also evolve O_2 from H_2O_2 in the light [(*2*, *8*) and data presented here)].

Two main results were reported in this study. First, it was found that there is no significant H_2O_2-dismutation activity from the dark S_1/S_{-1} cycle or the light-induced S_2/S_0 cycle of the O_2-evolving complex in photosystem II. The light-induced evolution of O_2 from H_2O_2 that was observed in both catalase-free PSII and Mn-depleted catalase-free PSII can be attributed to the disproportionation of superoxide formed via one-electron oxidation of hydrogen peroxide by $Y_Z^{\bullet}$. Second, micromolar concentrations of H_2O_2 significantly inactivated light-induced O_2 evolution in catalase-free photosystem II. Since PSII-mediated oxidation of H_2O_2 is catalyzed only in the light (eqs 1−4), the functional O_2-evolving complex would be susceptible to inactivation by exogenous H_2O_2 under dark conditions. It is notable that the PSII membrane-associated heme catalase is bound to the luminal surface of the thylakoid membrane and is active only above pH 6 (28). Because the pH of the lumen is acidic in the light, the photosystem II membrane-associated catalase may be crucial for protection of the O_2-evolving complex from damage by hydrogen peroxide during dark conditions.

REFERENCES

1. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
2. Velthuys, B., and Kok, B. (1978) *Biochim. Biophys. Acta 502*, 211−221.
3. Frasch, W. D., and Mei, R. (1987) *Biochemistry 26*, 7321−7325.
4. Frasch, W. D., and Mei, R. (1987) *Biochim. Biophys. Acta 891*, 8−14.
5. Frasch, W. D., Mei, R., and Sanders, M. A. (1988) *Biochemistry 27*, 3715−3719.
6. Fine, P. L., and Frasch, W. D. (1990) in *Current Research in Photosynthesis* (Baltscheffsky, M., Ed.) pp 905−908, Kluwer Academic Publishers, Dordrecht.
7. Fine, P. L., and Frasch, W. D. (1992) *Biochemistry 31*, 12204−12210.
8. Mano, J., Kawamoto, K., Dismukes, G. C., and Asada, K. (1993) *Photosynth. Res. 38*, 433−440.
9. Taoka, S., Jursinic, P. A., and Seibert, M. (1993) *Photosynth. Res. 38*, 425−431.
10. Mano, J., Takahashi, M., and Asada, K. (1987) *Biochemistry 26*, 2495−2501.
11. Beck, W. F., and Brudvig, G. W. (1987) *Biochemistry 26*, 8285−8295.
12. Kretschmann, H., and Witt, H. T. (1993) *Biochim. Biophys. Acta 1144*, 331−345.
13. Radmer, R. (1979) *Biochim. Biophys. Acta 546*, 418−425.
14. Yerkes, C. T., and Babcock, G. T. (1980) *Biochim. Biophys. Acta 590*, 360−372.
15. Hoganson, C. W., Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1989) *Photosynth. Res. 22*, 285−294.
16. Schröder, W. P., and Åkerlund, H.-E. (1986) *Biochim. Biophys. Acta 848*, 359−363.
17. Berg, S. P., and Seibert, M. (1987) *Photosynth. Res. 13*, 3−17.
18. Wydrzynski, T., Ångström, J., and Vänngård, T. (1989) *Biochim. Biophys. Acta 973*, 23−28.
19. Klimov, V., Ananyev, G., Zastryzhnaya, O., Wydrzynski, T., and Renger, G. (1993) *Photosynth. Res. 38*, 409−416.
20. Hillier, W., and Wydrzynski, T. (1993) *Photosynth. Res. 38*, 417−423.
21. Putrenko, I. I. (1996) *Biochemistry 35*, 2865−2871.
22. Wydrzynski, T., Hillier, W., and Messinger, J. (1996) *Physiol. Plant. 96*, 342−350.
23. Schröder, W. P., and Åkerlund, H.-E. (1990) in *Current Research in Photosynthesis* (Baltischeffsky, M., Ed.) pp 901−904, Kluwer Academic Publishers, Dordrecht.
24. Thompson, L. K., Blaylock, R., Sturtevant, J. M., and Brudvig, G. W. (1989) *Biochemistry 28*, 6686−6695.
25. Bader, K. P., and Schmid, G. H. (1990) *Z. Naturforsch. 45*, 757−764.
26. Bradley, R. L., Long, K. M., and Frasch, W. D. (1991) *FEBS Lett. 286*, 209−213.
27. Frasch, W. D. (1992) in *Manganese Redox Enzymes* (Pecoraro, V. L., Ed.) pp 47−70, VCH Publishers, New York.

28. Sheptovitsky, Y. G., and Brudvig, G. W. (1996) *Biochemistry 35*, 16255−16263.
29. Sandusky, P. O., and Yocum, C. F. (1988) *Biochim. Biophys. Acta 936*, 149−156.
30. Miyao, M., Ikeuchi, M., Yamamoto, N., and Ono, T. (1995) *Biochemistry 34*, 10019−10026.
31. Wardman, P., and Candeias, L. P. (1996) *Radiat. Res. 145*, 523−531.
32. Beck, W. F., de Paula, J. C., and Brudvig, G. W. (1985) *Biochemistry 24*, 3035−3043.
33. Berthold, D. A., Babcock, G. T., and Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
34. Arnon, D. I. (1949) *Plant Physiol. 24*, 1−15.
35. Buser, C. A., Diner, B. A., and Brudvig, G. W. (1992) *Biochemistry 31*, 11449−11459.
36. Tamura, N., and Cheniae, G. (1987) *Biochim. Biophys. Acta 890*, 179−194.
37. Ono, T., and Inoue, Y. (1983) *FEBS Lett. 164*, 255−260.
38. Margoliash, E., Novogorodsky, A., and Schejter, A. (1960) *Biochem. J. 74*, 339−348.
39. Miyao, M., and Inoue, Y. (1991) *Biochim. Biophys. Acta 1056*, 47−56.
40. Sheptovitsky, Y. G. (1997) Ph.D. Thesis, Yale University, New Haven, CT.

BI972872S