Oxygen Evolution from Hydrogen Peroxide in Photosystem II: Flash-Induced Catalatic Activity of Water-Oxidizing Photosystem II Membranes†

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ABSTRACT: Water-oxidizing photosystem (PS) II membranes treated with hydrogen peroxide in the dark showed backward transition of their S_0 state to the S_{-1} state, as deduced from the pattern of flash-dioxygen yields. In the presence of hydrogen peroxide, they evolved oxygen after the first flash, as reported for thylakoids [Velthuys, B., & Kok, B. (1978) *Biochim. Biophys. Acta 502*, 211–221]. We used $H_2{}^{18}O_2$ and a mass spectrometer that allowed monitoring of the oxygen isotopes in the medium to determine the flash yields. PS II membranes evolved $^{18}O_2$ and $^{16}O_2$ in $H_2{}^{18}O_2$ below 1 mM, but little $^{16}O_2$ was evolved above 1 mM. No $^{16}O^{18}O$ was evolved under any conditions. The oxygen yields from hydrogen peroxide at higher concentrations were more than 20-fold that for the PS II reaction center, and the decay of oxygen evolution after the flash ($t_{1/2} \sim 10$ s) was much slower than that from water. By shortening the dark interval between flashes, the evolution of $^{18}O_2$ was decreased and that of $^{16}O_2$ increased. Little $^{18}O_2$ was evolved under continuous light. The flash-induced evolution of dioxygen from hydrogen peroxide in PS II membranes is inferred from the assumption that the S_0/S_2-state cycle has catalatic activity with a slow rate and that this activity is lost by the reduction of S_0 to the S_{-1} state, the spontaneous decay of S_2 to the S_1 state, and the advancement of S_2 to the S_3 state by light. Tris(hydroxymethyl)aminomethane (Tris) treatment of PS II membranes decreased the yield of dioxygen and extinguished the kinetic features that characterize the catalatic production of dioxygen from hydrogen peroxide. In Tris-treated membranes, the oxygen yield was decreased by ascorbate but restored by the addition of superoxide dismutase, indicative of the production of superoxide by the univalent oxidation of hydrogen peroxide by the S_0 state.

Photosynthetic evolution of dioxygen is the four-equivalent oxidation of water. A cyclic scheme for the oxidation steps of the water-oxidizing enzyme, "S-state model", has been proposed by Kok et al. (1970). Little, however, is known about the chemical identity of this enzyme or of the interaction of water with the enzyme and oxygenated intermediates.

Many attempts to identify the water-oxidizing enzyme have been made by using oxygen-evolving photosystem (PS)[1] II complexes consisting of several polypeptides (Tang & Satoh, 1985; Satoh et al., 1985; Ikeuchi et al., 1985; Ghanotakis & Yocum, 1986). The 33-kDa protein has been suggested to have a key function in photosynthetic oxygen evolution, probably by stabilizing Mn binding on the active site of the enzyme (Miyao & Murata, 1984).

H_2O analogues such as NH_2OH (Radmer & Ollinger, 1982) and NH_2NH_2 (Hanssum & Renger, 1985) have been shown to affect the flash yield of dioxygen evolved from water and to be oxidized to N_2 at the site for the H_2O oxidation (Radmer & Ollinger, 1983). Hydrogen peroxide also is an analogue of water and has been reported to be an electron donor to the PS II of Tris-treated (Inoué & Nishimura, 1971) or NH_2OH-treated (Pan & Izawa, 1979) thylakoids. Velthuys and Kok (1978) showed that in the dark hydrogen peroxide reduces the S_1 and S_2 states to the "S_{-1}" and S_0 states of spinach thylakoids and suggested a "flash-induced catalase-like activity" of the thylakoids.

We have distinguished between dioxygen evolved from water and from hydrogen peroxide in the water-oxidizing PS II membranes after flash illumination. $H_2{}^{18}O_2$ and a mass spectrometric system which allows monitoring of dioxygen isotopes in the reaction medium were used. We have provided direct evidence of dioxygen production from hydrogen peroxide and of competition between hydrogen peroxide and water for the photooxidant generated in PS II. We also show that the water-oxidizing PS II membranes can catalyze the disproportionation of hydrogen peroxide into dioxygen and water after a single flash illumination but the Tris-treated ones cannot. The mechanism for the catalatic activity of PS II membranes is discussed.

Materials and Methods

A mass spectrometer system was constructed for the direct determination of dissolved gasses in solutions that was based on the system of Hoch and Kok (1963). We used a quadrupole mass analyzer (MSQ 150A; ULVAC, Japan) and an oil diffusion pump (YDP-04; ULVAC, Japan). A gas-permeable membrane (24 μm thick, silicone rubber, General Electric, or 100 μm thick, Teflon, Chukoh Kasei Co., Ltd., Japan) was placed at the bottom of the reaction vessel to separate the medium from the high-vacuum inlet to the analyzer. The reaction vessel cylinder (1 cm in diameter) was made of glass and was maintained at 25 °C by circulating water through both the cylinder and the stainless-steel base. The cylinder was covered by a piston lid made of acrylic resin.

Thylakoids were prepared from market spinach leaves and their oxygen-evolving PS II membranes with Triton X-100

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[1] Abbreviations: Chl, chlorophyll; Cyt *c*, cytochrome *c*; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; DETAPA, diethylenetriaminepentaacetic acid; DPC, diphenyl carbazide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SOD, superoxide dismutase; PS, photosystem; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s).

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according to Kuwabara and Murata (1982). The PS II activity of the PS II membranes was more than 290 μmol of oxygen evolved (mg of Chl)$^{-1}$ h^{-1} when *p*-benzoquinone was the electron acceptor. PS I activity, however, was less than 20 μmol (mg of Chl)$^{-1}$ h^{-1} in a reaction mixture containing 0.5 mM ascorbate, 40 μM DCIP, 0.1 mM methylviologen, 1 mM NaN_3, 10 μM DCMU, and 50 mM phosphate buffer, pH 7.0. PS II membranes were prepared from wheat leaves with Triton X-100 according to Ono and Inoue (1983). The PS II activity of the wheat PS II membranes was more than 400 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. Tris treatment of the thylakoids and of the PS II membranes was done by incubating samples (0.5 mg of Chl mL^{-1}) for 20 min with stirring under room light at 4 °C in 0.8 M Tris-HCl, pH 8.0. NaCl treatment (Miyao & Murata, 1983) and Cl^- depletion (Itoh et al., 1984) of PS II membranes were done to inactivate water-oxidizing activity. The thylakoids and PS II membranes were suspended in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 20 mM Mes–NaOH, pH 6.5, at 1–3 mg of Chl mL^{-1}.

For the flash illuminations, a 10-μL suspension of PS II membranes (with 0.3% digitonin) or thylakoids was gently layered on the silicone rubber membrane at the bottom of the reaction vessel which contained 0.4 mL of medium (50 mM Hepes, pH 6.8, 1 mM NaN_3, and $H_2{}^{16}O_2$ or $H_2{}^{18}O_2$ where indicated) which had been flushed with Ar gas. Azide was indispensable to avoid decomposition of the hydrogen peroxide by contaminating catalse in the thylakoids and PS II membranes. An addition of digitonin to PS II membranes was necessary to spread them uniformly over the bottom membrane. DCMU and tetranitromethane were included in the membrane suspension. The samples then were incubated in the dark for 5 min, during which time the components of the upper medium (hydrogen peroxide and azide) were diffused to the sample suspension. This was confirmed by the same yield of oxygen being obtained from water or hydrogen peroxide, whether or not the sample suspension contained the components of the upper medium. After a 5-min dark incubation, the samples are illuminated by a xenon flash generator (30 J, $t_{1/2}$ = 30 μs; Sugawara Kenkyusho Co., Ltd., Japan) set directly above the reaction vessel. Under these conditions, the half-decay time of the signal of the oxygen evolved from water after a flash in the 0.13-mm-thick sample layer was 2 s.

Oxygen evolution under continuous light was measured with a Clark-type oxygen electrode (Hansatech Co. Ltd., England) or with our mass spectrometer vessel equipped with a Teflon membrane. The reaction mixture (1 mL) contained PS II membranes (10–30 μg of Chl), 10 mM $MgCl_2$, 0.3 mM *p*-benzoquinone, 1 mM NaN_3, and 20 mM Hepes–NaOH, pH 6.8, with or without H_2O_2 (or $H_2{}^{18}O_2$) at the indicated concentrations. White actinic light from the tungsten lamp or a projector was passed through a water filter. The light intensity was 83.3 mW cm^{-2}.

$H_2{}^{18}O_2$ was synthesized according to Asada and Badger (1984) with slight modifications: 3 mL of $^{18}O_2$ gas was introduced to 20 mL of an evacuated reaction mixture containing 200 mM glucose, 0.5 mM EDTA, 0.5 mM KCN, 0.01% glucose oxidase (Sigma Chemical Co.), and 10 mM phosphate buffer, pH 6.0. After incubation at 25 °C for 5 h, the reaction was terminated by adding 0.4 mL of 1 M HCl, after which the solution was neutralized with KOH. CN^- was removed through a AG 1-X8, Cl^- form column (Bio-Rad), and the product was stored at −20 °C until use. The ^{18}O content of the hydrogen [^{18}O]peroxide was 81% based on the ratio of $^{18}O_2/^{16}O_2$ evolved after the addition of catalase to the mass spectrometer vessel. Mn-SOD from *Serratia marcesens* (Wako Pure Chemical Industries, Ltd., Japan) was used because it is not inactivated by hydrogen peroxide (Asada et al., 1975).

Results

Reduction of S States by Dark Incubation with Hydrogen Peroxide. Spinach thylakoids first were treated with 3.3 mM H_2O_2 for 5 min at pH 8.8 and then depleted of H_2O_2 by catalase, and the pH was lowered to 7.8. After incubation in the dark for 5 min, the pattern of flash-oxygen evolution was delayed by two flashes compared to that in the untreated thylakoids as reported by Velthuys and Kok (1978). Adopting their hypothesis that the S_{-1} state, which would be oxidized to the S_0 state by a single flash, is formed by hydrogen peroxide treatment, we made an approximation on the assumption that the miss-hit (0.19) and double-hit (0.06) constants for the untreated thylakoids were not affected by H_2O_2/catalase treatment. The best fit was obtained when fractions of the S_0, S_{-1}, and S_1 states were 0%, 59%, and 41%, respectively, whereas in the untreated thylakoids the fraction of the S_0 state was 21% and the S_1 state 79% (data not shown). Complete reduction of the S_1 to the S_{-1} state as reported by Velthuys and Kok (1978) was not found. Extension of the dark incubation period after H_2O_2/catalase treatment of 15 min did not affect the pattern of flash-oxygen evolution, evidence of the stability of the S_{-1} state.

When wheat PS II membranes were treated with H_2O_2/catalase, we observed a delay of flash-oxygen evolution similar to that with spinach thylakoids. Incubation in 30 mM H_2O_2 at pH 8.8 followed by H_2O_2 removal with catalase produced the S_0, S_{-1}, and S_1 state fractions of 0%, 56%, and 44%, respectively. The S_{-1} fraction was 64% even when the membranes were treated with 120 mM H_2O_2 for 3 min.

To determine whether the dioxygen evolved after the treatment with H_2O_2/catalase was derived from hydrogen peroxide or water, we treated spinach thylakoids with 30 mM $H_2{}^{18}O_2$ at pH 8.8 for 1 min followed by its removal by catalase and lowering of the pH to 7.8. All the dioxygen evolved after flash illuminations was $^{16}O_2$. Neither $^{18}O_2$ nor $^{16}O^{18}O$ was evolved (data not shown). Thus, hydrogen peroxide did not bind to, or was easily released from, the water-oxidizing enzyme but worked as a reductant. The backward shift of the S state with hydrogen peroxide is similar to that with NH_2OH (Radmer & Ollinger, 1982) or NH_2NH_2 (Hanssum & Renger, 1985).

Dioxygen Is Formed from Hydrogen Peroxide by PS II Membranes. In the presence of hydrogen peroxide, dioxygen is evolved by spinach thylakoids after the first flash (Velthuys & Kok, 1978). We found that this also was true for spinach and wheat PS II membranes. To determine whether the dioxygen evolved by a flash illumination in the presence of hydrogen peroxide comes from water or hydrogen peroxide, we flash illuminated spinach thylakoids as well as PS II membranes in the presence of 0.2–3.0 mM $H_2{}^{18}O_2$. Thylakoids and PS II membranes gave similar results which are shown in Figure 1. In the presence of $H_2{}^{18}O_2$, both $^{18}O_2$ and $^{16}O_2$ were evolved, but no $^{16}O^{18}O$ was detected. These results indicate that the dioxygen evolved in thylakoids and PS II membranes after the first flash is derived from hydrogen peroxide but that no scrambling of oxygen atoms from $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$ or from $H_2{}^{16}O$ and $H_2{}^{18}O_2$ occurs. Because the results for thylakoids and PS II membranes were the same, the photoevolution of dioxygen from hydrogen peroxide must be an intrinsic reaction of PS II but not one of PS I or thylakoid-bound catalase.

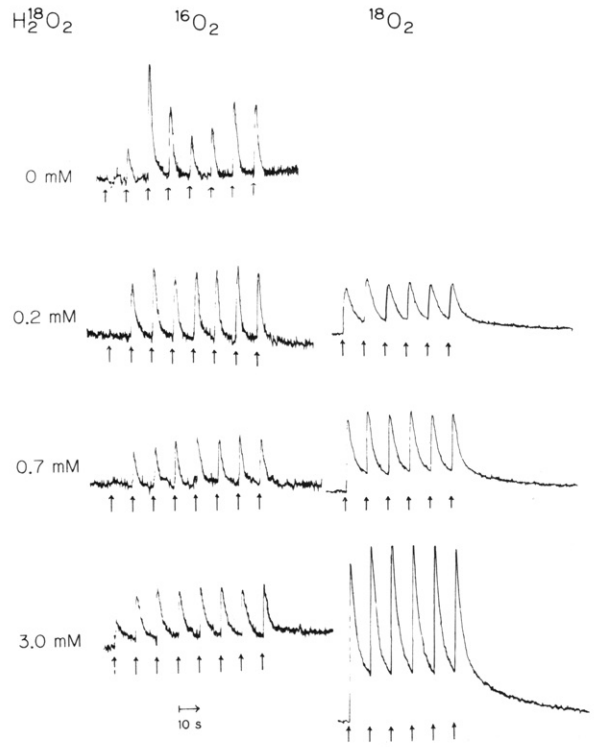

FIGURE 1: Evolution of $^{16}O_2$ and $^{18}O_2$ from spinach PS II membranes in various concentrations of $H_2^{18}O_2$ after sequential flash illuminations. Spinach PS II membranes (10 μL in 0.4 M sucrose, 5 mM $MgCl_2$, 20 mM Hepes–NaOH, pH 6.5, and 0.3% digitonin) were layered on the bottom of the mass spectrometer vessel which contained 0.4 mL of 50 mM Hepes–NaOH, pH 6.8, 1 mM NaN_3, and $H_2^{18}O_2$ at the indicated concentration. The membranes then were dark adapted for at least 5 min. The evolutions of $^{18}O_2$ (m/e 36), $^{16}O^{18}O$ (m/e 34), and $^{16}O_2$ (m/e 32) were followed with six or eight sequential flashes spaced 10 s apart as indicated by the arrows. No $^{16}O^{18}O$ was evolved at any of the concentrations of $H_2^{18}O_2$ tested.

Above 1 mM $H_2^{18}O_2$, little $^{16}O_2$ was evolved from water. At low concentrations of $H_2^{18}O_2$, the flash yield pattern of $^{16}O_2$ was very similar to that for dioxygen evolution from water, the maximum yield coming after the third flash. By contrast, the pattern of the flash yield of $^{16}O_2$ in 3 mM $H_2^{18}O_2$ was the same as that for the dioxygen evolved from hydrogen peroxide, evolution of dioxygen from the first flash being due to the evolution of $^{16}O_2$ from $H_2^{16}O_2$ in the hydrogen [^{18}O]peroxide used (Figure 1). The yields of dioxygen from hydrogen peroxide and water produced by the first six flashes were compared for various concentrations of $H_2^{18}O_2$. The flash yield of $^{18}O_2$ increased with an increase in the $H_2^{18}O_2$ concentration, but the yield of $^{16}O_2$ from water decreased (Figure 2). Thus, water and hydrogen peroxide appear to compete for the photooxidant generated in PS II.

Some PS II membrane preparations required electron acceptors in PS II for the full extent of the flash evolution of dioxygen from water. However, when we used spinach PS II membranes whose flash yield of oxygen from water in the absence of acceptors was 9% of that in the presence of 5 mM ferricyanide, they produced dioxygen from 10 mM hydrogen peroxide at the equivalent yield regardless of whether the acceptor was present. These results suggest that hydrogen peroxide or superoxide produced from hydrogen peroxide (described later) may also act as an electron acceptor in PS II.

The flash evolution of oxygen from hydrogen peroxide by spinach thylakoids is not completely inhibited by DCMU (Velthuys & Kok, 1978). This also was true for spinach PS II membranes. The remaining flash yields of oxygen from 1

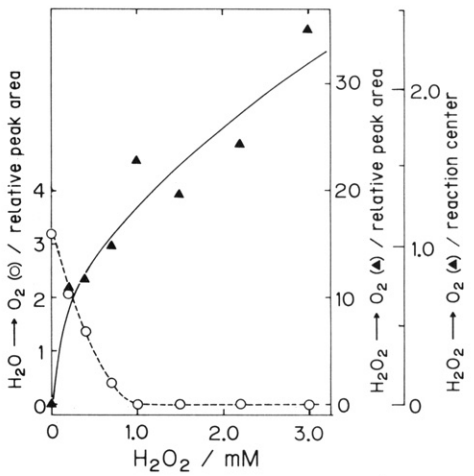

FIGURE 2: Inhibition of the photooxidation of water by spinach PS II membranes with hydrogen peroxide after flash illumination. The flash yields of $^{18}O_2$ and $^{16}O_2$ from spinach PS II membranes in the presence of $H_2^{18}O_2$ were determined as in Figure 1. The values for the evolution of dioxygen from water and hydrogen peroxide produced by the first six flashes were separately totaled and are shown by the relative peak area. The $^{16}O/^{18}O$ ratio in hydrogen [^{18}O]peroxide was 19/81. Therefore, the oxygen yields from water and hydrogen peroxide were corrected as follows: $^{16}O_2$ evolution from $H_2^{16}O_2$ in hydrogen [^{18}O]peroxide was $[^{18}O_2] \times 19/81$; for the dioxygen from water (open circles), $[^{16}O_2] - [^{18}O_2] \times 19/81$; dioxygen evolved from hydrogen peroxide (closed triangles), $[^{18}O_2] \times 100/81$. For the estimation of the PS II reaction center, see Figure 5.

mM H_2O_2 produced by the first, second, and third flashes were 69%, 54%, and 57%, respectively, of the control value with DCMU, even at 2.5 mM. Under the same conditions, no flash evolution of dioxygen from water was found in the PS II membranes. A similar, incomplete inhibition of the flash yield of oxygen from hydrogen peroxide took place in Tris-treated spinach PS II membranes. This incomplete inhibition of oxygen evolution by DCMU is accounted for by the assumption that hydrogen peroxide or superoxide can accept electron(s) from PS II at a site(s) before the Q_B site at which DCMU blocks electron transport.

Prolonged Evolution of Dioxygen from Hydrogen Peroxide. Dioxygen evolution from hydrogen peroxide by water-oxidizing spinach PS II membranes continued longer than that from water (Figure 1). A similar prolonged oxygen evolution from hydrogen peroxide has been reported for spinach thylakoids (Velthuys & Kok, 1978). The decay curves ($t_{1/2} \sim 10$ s) approximated second or higher order kinetics when the concentration of hydrogen peroxide was less than 30 mM, evidence that two or more components determine the decay kinetics. In 180 mM hydrogen peroxide, the decay kinetics appeared to be first-order kinetics (data not shown).

The prolonged oxygen evolution after the flash indicates that the dioxygen yield from hydrogen peroxide depends on the dark period between flashes. The effect of the dark interval on the ratio of dioxygen from water to that from hydrogen peroxide evolved by 12 flashes shows that this expectation is met (Figure 3). The shorter the dark interval between flashes, the higher the dioxygen yield from water and the lower that from hydrogen peroxide. The total yields of oxygen from both hydrogen peroxide and water also decreased when the flash interval was reduced. Because oxygen is evolved from water with a half-time that is within 12 ms after the flash in PS II membranes (Boussac et al., 1985), the change in the dark period in our experiments should have affected only oxygen evolution from hydrogen peroxide, not that from water.

Under continuous illumination, oxygen should be evolved from water exclusively even in the presence of hydrogen

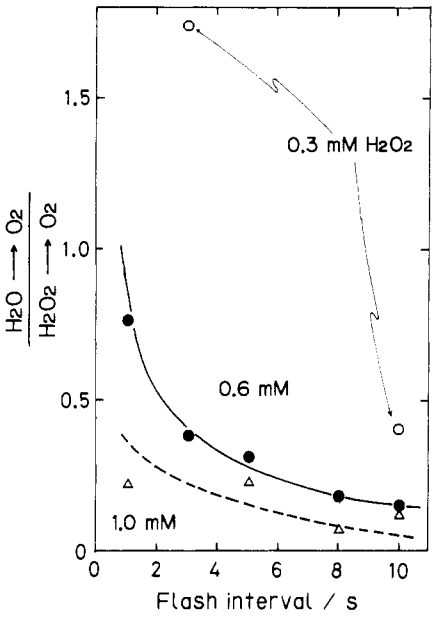

FIGURE 3: Effects of the interval between flashes on the ratios of oxygen evolved from water and from hydrogen peroxide in spinach PS II membranes at various concentrations of $H_2{}^{18}O_2$. Experimental conditions were the same as in Figure 2 except for the varied intervals between flashes. Hydrogen [^{18}O]peroxide was added at the concentrations indicated. The relative oxygen yields from water and hydrogen peroxide produced by 12 flashes were determined as in Figure 2.

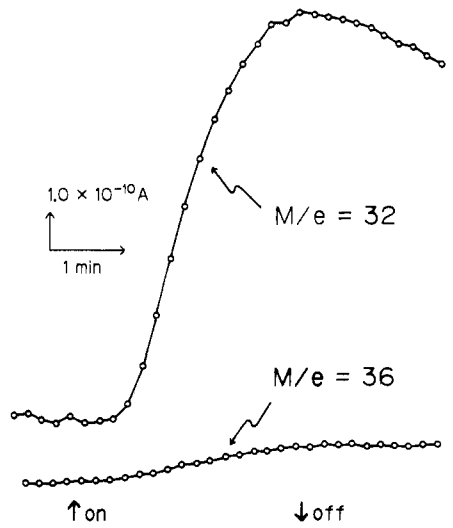

FIGURE 4: $^{18}O_2$ and $^{16}O_2$ evolution by spinach PS II membranes in 3 mM $H_2{}^{18}O_2$ under continuous illumination. The reaction mixture (1 mL) contained spinach PS II membranes (30 μg of Chl), 10 mM $MgCl_2$, 1 mM NaN_3, 0.3 mM *p*-benzoquinone, 3 mM $H_2{}^{18}O_2$, and 20 mM Hepes–NaOH, pH 6.8, and was stirred in the Teflon membrane suited reaction vessel during the measurement of $^{18}O_2$, $^{16}O_2$, and $^{16}O^{18}O$. No $^{16}O^{18}O$ was evolved.

peroxide. The results in Figure 5 indicate that this was the case. PS II membranes in 3 mM $H_2{}^{18}O_2$ evolved little $^{18}O_2$, and most of the dioxygen evolved was $^{16}O_2$ derived from water (Figure 4), whereas no dioxygen was evolved from water when flashes spaced at 10 s were given in the same concentration of $H_2{}^{18}O_2$ (Figure 2).

Catalatic Activity in PS II Is Induced by a Flash. Unlike oxygen evolution from water, that from hydrogen peroxide took place after the first flash (Figure 1). If hydrogen peroxide is oxidized univalently by the PS II reaction center's chlorophyll cation generated by one flash, the superoxide radical will be produced. Therefore, the yield of dioxygen evolved through

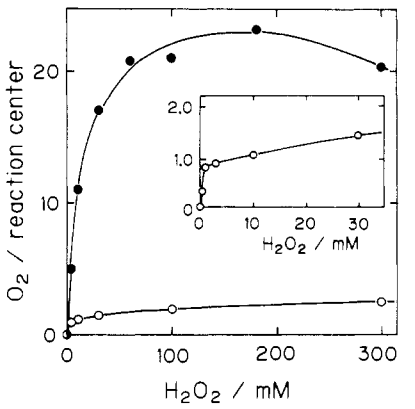

FIGURE 5: Yield of oxygen from hydrogen peroxide after a single flash to the control and Tris-treated spinach PS II membranes. Control or Tris-treated spinach PS II membranes (10 μL, 3.0 mg of Chl mL^{-1}) were placed on the bottom of a vessel filled with 0.4 mL of medium that contained 10 mM NaN_3, 50 mM Hepes–NaOH, pH 6.8, and various concentrations of H_2O_2. After dark incubation for 5 min, the oxygen yield produced by a single flash was measured, and the ratio of the dioxygen yield from hydrogen peroxide by a flash to the PS II reaction center (O_2/reaction center) was determined. Because dioxygen evolution from water at 10-s spaced flashes was completely inhibited by H_2O_2 at more than 1 mM in PS II membranes (Figure 2) and in Tris-treated membranes, all the $^{16}O_2$ evolved was assumed to be derived from hydrogen peroxide. The content of the reaction center in PS II membranes was estimated from the equation Y_{ss} (moles of O_2) = (1/4)(1 − α)(reaction center) (moles) in which Y_{ss} and α are the steady-state level of the flash-O_2 yield and the miss-hit constant determined by fitting the oxygen yields produced by eight flashes to Kok's scheme with the fixed condition (S_0/S_1) being 1/3. It was assumed that α was not affected by hydrogen peroxide. Inset: Dioxygen evolution from hydrogen peroxide by Tris-treated PS II membranes at low concentrations of hydrogen peroxide. The ordinate represents the O_2 per reaction center. (Closed circles) Control PS II membranes; (open circles) Tris-treated PS II membranes.

the disproportionation of superoxide should be 0.5 mol per reaction center.

$$H_2O_2 \xrightarrow{Chl^+\ Chl} O_2^- + 2H^+ \quad (1)$$

$$O_2^- + H^+ \longrightarrow 0.5O_2 + 0.5H_2O_2 \quad (2)$$

The dioxygen yield from hydrogen peroxide in PS II membranes was, however, higher than 0.5 mol per reaction center when the concentration of hydrogen peroxide was more than 0.5 mM (Figure 2). The number of reaction centers in PS II membranes was estimated by assuming that a quarter of the centers equals the steady-state flash yield of oxygen from water (Y_{ss}). Y_{ss} was calculated from Kok's S-state model (Kok et al., 1970) based on the oxygen yield pattern by the first eight flashes in the absence of hydrogen peroxide. With a hydrogen peroxide concentration of more than 100 mM, the dioxygen evolved after the first flash was more than 20-fold the amount for the reaction centers (Figure 5).

The high yield of dioxygen from hydrogen peroxide may be attributable to a finite multiple turnover of the enzyme for catalatic activity in PS II that is "triggered" by a single flash. During the dark reaction after a flash, the active state of the PS II membranes would shift to the "resting state" for catalatic activity, and oxygen evolution from the hydrogen peroxide would decrease gradually. Membranes in the resting state would again become active when the next flash is given. Such catalatic activity is not attributable to metal ions present in the samples or the medium because the addition of EDTA or DETAPA had no effect on the oxygen yield (Table I).

A slight decrease in dioxygen yield at higher concentrations of hydrogen peroxide than 100 mM is thought to be caused

Table I: Effects of Reagents That React with Superoxide Anion on the Flash-Oxygen Yield from Hydrogen Peroxide by Spinach PS II Membranes[a]

addition	concn	control	Tris treated
none		100	100
Tiron	1.0 mM	100	91
	3.0 mM	100	42
ascorbate	1.0 mM	100	23
+Mn-SOD	0.1 μM		31
+Mn-SOD	1.0 μM		48
Mn-SOD	1.1 μM	106	78
tetranitromethane	0.1 mM	112	106
Cyt c (Fe^{3+})	40 μM		101
EDTA	0.5 mM	112	77
DETAPA	0.5 mM	103	76

[a] Control or Tris-treated spinach PS II membranes containing the indicated reagent(s) were dark adapted in 10 mM H_2O_2 as in Figure 1. The flash-oxygen yields produced by 12 flashes are shown as relative values. The dioxygen yield produced by Tris-treated PS II membranes alone was 18% of that produced by the control PS II membranes (Figure 5).

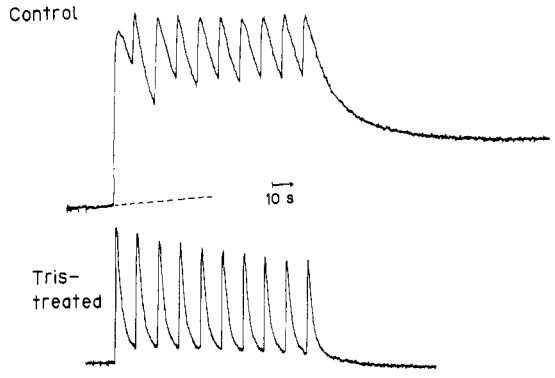

FIGURE 6: Time course of oxygen evolution from hydrogen peroxide by the control and Tris-treated spinach PS II membranes after flash illumination. Suspensions of control or Tris-treated spinach PS II membranes (10 μL, 1 mg of Chl mL^{-1}) were placed on the bottom of a reaction vessel that contained 0.4 mL of 10 mM H_2O_2, 1 mM NaN_3, and 20 mM Hepes–NaOH, pH 6.8. After dark incubation for 5 min, sequential flashes were given every 10 s.

by the inactivation of the water-oxidizing enzyme and the PS II reaction center by hydrogen peroxide in the dark. Photoreduction of DCIP by PS II membranes in the presence and in the absence of DPC was inhibited by 16% and 12%, respectively, in 5 min with 3 mM H_2O_2 in the dark. The flash-induced catalatic activity of PS II membranes is lost by treatment which inhibits oxygen evolution from water. Tris-treated spinach PS II membranes evolved $^{18}O_2$ from $H_2{}^{18}O_2$ but no $^{16}O_2$ from water under continuous light or flashes (data not shown); therefore, Tris-treated PS II membranes do not photooxidize water, as expected, but can photooxidize hydrogen peroxide even under continuous light.

The flash-oxygen yield pattern of Tris-treated PS II membranes in the presence of 10 mM hydrogen peroxide was similar to that of the control membranes; oxygen was evolved after the first flash (Figure 6). This also was true for spinach thylakoids (Velthuys & Kok, 1978). However, unlike the water-oxidizing PS II membranes, for Tris-treated membranes the flash-oxygen yield from hydrogen peroxide was very low. The yield was saturated at about 1 mM hydrogen peroxide, and the ratio of the yield to the PS II reaction center was nearly 1 (Figure 5, inset). The gradual increase in the yield above 1 mM hydrogen peroxide is attributable to the flash-induced catalatic activity of the water-oxidizing enzyme remaining active after Tris treatment.

In addition to a low yield of oxygen from hydrogen peroxide, the decay of oxygen evolution after the flash and the oscillation of both the flash yield and the decay rate by a peroxide of two flashes were greatly affected by Tris treatment. In Tris-treated membranes, the decay rate of oxygen evolution after the first flash ($t_{1/2}$) decreased to 3 s and binary oscillation disappeared (Figure 6), which agrees with results found for thylakoids (Velthuys & Kok, 1978).

When the water-oxidizing activity of wheat PS II membranes was inactivated by treatment with 1 M NaCl (Miyao & Murata, 1983) or by depletion of Cl^- (Itoh et al., 1984), flash-oxygen yield from hydrogen peroxide was decreased, the decay accelerated, and the binary oscillations diminished as in Tris-treated ones (data not shown). These results provide further evidence that the flash-induced catalatic activity is due to the water-oxidizing enzyme.

Superoxide Is an Intermediate of the Oxygen Evolved from Hydrogen Peroxide in Tris-Treated PS II Membranes. Superoxide is a probable intermediate in the evolution of oxygen from hydrogen peroxide (reactions 1 and 2). To determine whether superoxide actually is produced during the oxidation of hydrogen peroxide, we examined the effects of compounds that react with O_2^- on the flash-oxygen yield from hydrogen peroxide in the control and Tris-treated PS II membranes (Table I). In the Tris-treated PS II membranes, the oxygen yield was decreased by Tiron and ascorbate, which reduce O_2^- to H_2O_2 at 5×10^8 M^{-1} s^{-1} (Greenstock & Miller, 1975) and at 10^5 M^{-1} s^{-1} (Cabelli & Bielski, 1983). The inhibition of oxygen evolution from hydrogen peroxide by ascorbate was partially reversed by SOD, confirming the production of O_2^-.

When SOD alone was added, it inhibited oxygen evolution from hydrogen peroxide in Tris-treated membranes. If dioxygen was produced by the disproportionation of O_2^-, SOD would not affect the yield. This inhibition by SOD suggests that part of the superoxide was oxidized to dioxygen by an oxidant before its disproportionation; therefore, 1 mol of O_2, rather than 0.5 mol, could be generated from 1 mol of O_2^- (Figure 5). We have yet to identify the oxidant, but its content must be greater than six molecules per reaction center because inhibition of dioxygen evolution by SOD took place even at the sixth flash.

Tetranitromethane (McCord & Fridovich, 1969) and cytochrome c (Koppenol et al., 1976) oxidize O_2^- to O_2 at 2×10^9 and 1.1×10^6 M^{-1} s^{-1}. An addition of either compound had little effect on the yield (Table I). This is to be expected if an endogenous oxidant is sufficient for the oxidation of O_2^- to O_2.

The inhibitory effects of chelators on oxygen evolution in Tris-treated membranes indicate the participation of free metal ions in the reaction. Mn ions might mediate electron donation from hydrogen peroxide to the PS II reaction center, as shown for NH_2OH-treated (Velthuys, 1983) and NaCl-treated (Schröder & Åkerlund, 1986) PS II membranes. The oxidation of superoxide by hydrogen peroxide catalyzed by transition metal ions (metal-catalyzed Haber–Weiss reaction; Baker & Gebicki, 1984) is a possible reaction by which dioxygen could be produced from the superoxide.

Unlike with Tris-treated membranes, SOD, Tiron, and ascorbate had no effects on oxygen evolution from hydrogen peroxide in control PS II membranes (Table I). Therefore, O_2^- production through the oxidation of hydrogen peroxide and dioxygen formation through its disproportionation are unlikely in native PS II membranes.

Discussion

In dark-adapted thylakoids and PS II membranes, S_1 is the predominant state and S_0 a minor one. When thylakoids or PS II membranes were treated with hydrogen peroxide, S_0 was

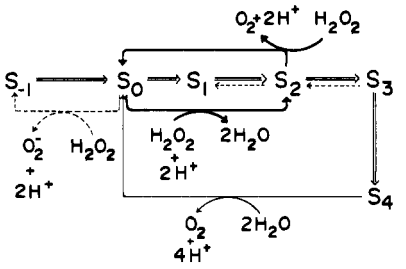

FIGURE 7: Model for possible interactions of hydrogen peroxide with the S states of the water-oxidizing enzyme.

shifted backward to S_{-1}. Reduction of S_0 by hydrogen peroxide affects the S_0/S_1 balance, and S_1 is reduced, so that a new equilibrium of the S_{-1} and S_1 states is reached. The S_{-1}/S_1 state is stable in the dark; dark incubation for 15 min after H_2O_2/catalase treatment did not affect the S_{-1}/S_1 ratio.

When a single flash is given to the S_{-1}/S_1 state of PS II membranes, it is shifted one step forward to the S_0/S_2 state. This state can evolve a multiple number of oxygen molecules from hydrogen peroxide per reaction center. Because no $^{16}O^{18}O$ was evolved under any conditions, dioxygen evolved from hydrogen peroxide by PS II membranes must originate from a single hydrogen peroxide molecule, though a reaction like that of catalase (Jarnagin & Wang, 1958) but distinct from that of chloroperoxidase, in which the dioxygen evolved is derived from two different molecules of hydrogen peroxide (Hager et al., 1972). We propose the following reaction mechanism of the flash-induced catalatic activity (Figure 7): S_0 is divalently oxidized and S_2 is divalently reduced by hydrogen peroxide, thereby respectively producing water and dioxygen. Once this S_0/S_2 catalytic cycle is set to act, it evolves a multiple number of dioxygen molecules per reaction center (Figure 5). The rate of oxidation of S_0 to S_2 by hydrogen peroxide must be much greater than that of reduction of S_0 to S_{-1} because little superoxide was detected (Table I).

The evolution rate of dioxygen from hydrogen peroxide through the S_0/S_2 catalatic cycle should be proportional to the fraction of the S_0 and S_2 states. The fraction of S_0 depends on the reduction rate to S_{-1}, the transition probability to S_1, and the S_2 reduction rate to S_0. The fraction of S_2 depends on the reduction rate to S_0, the transition probability to the S_3 state, and the S_0 oxidation rate to S_2. The reduction and oxidation rates are functions of the concentration of hydrogen peroxide, and the transition probabilities are those of the flash interval. Therefore, under a fixed flash interval, H_2O_2 at a high concentration produces a greater turnover of the S_0/S_2 cycle for the evolution of dioxygen from H_2O_2 and less chance for S_2 to be photooxidized beyond the S_3 state to evolve dioxygen from water (Figure 2). At a fixed H_2O_2 concentration, a short dark period between flashes decreases the chance for S_2 to be reduced by hydrogen peroxide but increases the chance for it to be photooxidized beyond the S_3 and S_4 states to evolve more dioxygen from water (Figure 3); under continuous light, oxygen is evolved almost exclusively from water, not from hydrogen peroxide (Figure 4).

The decay of the catalatic evolution of oxygen may be attributed to the dark decay of the S_2 to the S_1 state and the reduction of the S_0 to the S_{-1} state by hydrogen peroxide. After that, the next flash again sets off the catalatic cycle. The dark decay of the S_2 to the S_1 state and the reactivities of the S_2 and S_0 states with hydrogen peroxide appear to determine the population of the S_0/S_2 catalatic cycle. The dark decay rate of the S_2 state of the PS II membranes has been determined to be about 20 s (Seibert & Lavorel, 1983). The faster decay of the catalatic evolution of oxygen after the first flash ($t_{1/2}$ $\sim$ 10 s in 10 mM H_2O_2), therefore, might be due to the reduction of S_0 to S_{-1}.

If the next flash is given before the S_{-1}/S_1 state is reached, the S_1 and S_3 states will be formed by the univalent photooxidation of S_0 and S_2 in addition to the S_0 and S_2 states formed from respective preceding states. The resulting S_3 state would become able to oxidize water on the next flash illumination because the fraction of S_3 decaying to the S_2 state within 10 s is estimated to be very small (a half-time of about 100 s; Seibert & Lavorel, 1983). Thus, the fractions of the S_0 and S_2 states after the second flash are small compared to those after the first flash. This alteration in the each S-state population, dependent on the flash number, may cause the oscillation of the yield and the decay of flash-oxygen evolution from hydrogen peroxide by two flashes (Figure 6).

Tris treatment impairs the water-oxidizing activity of PS II membranes by releasing three peripheral proteins (18, 24, and 33 kDa) and Mn atoms (Murata et al., 1983; Yamamoto & Nishimura, 1983). For Tris-treated thylakoids and PS II membranes, the yields of dioxygen from hydrogen peroxide decreased, and the decay of dioxygen evolution accelerated. In this case, the S_0 state produced by the flash would not be oxidized to the S_2 state by hydrogen peroxide, because the ESR-detectable S_2 state has not been observed in Tris-treated thylakoids (Dismukes & Siderer, 1981). The S_0 state would be reduced univalently to the S_{-1} state by hydrogen peroxide. Resulting superoxide then is oxidized by an oxidant and produces 1 mol of dioxygen per reaction center on a single flash (Figure 5, inset). This oxygen production via superoxide is terminated early and is seen as having a greater decay rate than that of catalatic oxygen evolution in untreated membranes. Because the addition of chelators decreased the yield of dioxygen, free Mn ions appeared to participate in the reaction by Tris-treated membranes but did not by the water-oxidizing ones, where chelators did not affect the yield (Table I).

The structural resemblance of H_2O_2 to H_2O–H_2O and the "crypto–peroxi" complex as a possible intermediate during the oxidation of water to dioxygen (Renger et al., 1983) has led us to suppose that the site of the H_2O_2 redox reaction is the H_2O binding active center of the water-oxidizing enzyme. This center would also bind to and oxidize NH_2OH because the effects of NH_2OH on the reduction of S states (Radmer & Ollinger, 1982) are very similar to those of H_2O_2 and the pattern of the flash yield of N_2 from NH_2OH (Radmer & Ollinger, 1982) is similar to that of O_2 from H_2O_2. Furthermore, the distance between oxygen atoms in H_2O_2 (1.464 Å; Bair & Goddard, 1982) agrees well with the size proposed for the binding site of H_2O (Radmer & Ollinger, 1983).

The inhibition of H_2O oxidation by hydrogen peroxide (Figure 2), however, should not be interpreted as a direct competition between these two substrates for the same S_2 state. This is because hydrogen peroxide reacts with the S_0 and the S_2 states while H_2O molecules in the water-oxidizing enzyme in S states smaller than S_3 are exchangeable with those in bulk medium (Radmer & Ollinger, 1986). Probably S states beyond S_2 coordinate H_2O (Hansson et al., 1986), and the S_4 state may have a redox potential high enough to oxidize water to dioxygen (0.81 V; Fee & Valentine, 1977) whereas the redox potential in the S_2 state might be sufficient to oxidize hydrogen peroxide.

Although the component(s) that catalyze(s) the flash-induced catalatic reaction is (are) still unclear, it would be appropriate to suppose that the water-oxidizing enzyme has this activity. A manganese-containing, 12-kDa protein from

the cyanobacterium *Plectonema boryanum* shows a catalatic activity, and the antibody against that protein inhibits electron transport in the oxidizing side of PS II (Okada & Asada, 1983). A catalatic activity has been found for cytochrome *c* oxidase (Orii & Okunuki, 1963) which catalyzes the 4 equiv reduction of dioxygen to water, the reverse reaction of photosynthetic oxygen evolution from water. Recently, Takahashi et al. (1986) showed that the Q_B binding D1 protein was labeled with iodine which was photooxidized by PS II and suggested this protein to be located on the donor side. It is interesting whether D1 protein holds any H_2O_2 binding sites.

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