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Photoproduction of Catalase-Insensitive Peroxides on the Donor Side of Manganese-Depleted Photosystem II: Evidence with a Specific Fluorescent Probe

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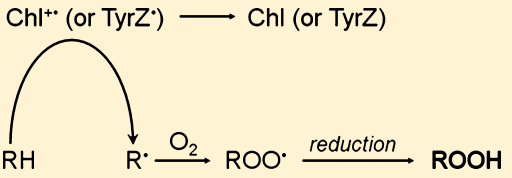
ABSTRACT: The photoproduction of organic peroxides (ROOH) in photosystem II (PSII) membranes was studied using the fluorescent probe Spy-HP. Two types of peroxide, highly lipophilic ones and relatively hydrophilic ones, were distinguished by the rate of reaction with Spy-HP; the former oxidized Spy-HP to the higher fluorescent form Spy-HPOx within 5 min, while the latter did so very slowly (the reaction was still not completed after 180 min). The level of photoproduction of these peroxides was significantly larger in the alkaline-treated, Mn-depleted PSII membranes than that in the untreated membranes, and it was suppressed by an artificial electron donor (diphenylcarbazide or ferrocyanide) and by the electron transport inhibitor diuron. Postillumination addition of Fe^{2+} ions, which degrade peroxides by the Fenton mechanism, abolished the accumulation of Spy-HPOx, but catalase did not change the peroxide level, indicating that the detected species were organic peroxides, excluding H_2O_2. These results agreed with our previous observation of an electron transport-dependent O_2 consumption on the PSII donor side and indicated that ROOH accumulated via a radical chain reaction that started with the formation of organic radicals on the donor side. Illumination ($\lambda > 600$ nm; 1500 μmol of photons m^{-2} s^{-1}) of the Mn-depleted PSII membranes for 3 min resulted in the formation of nearly 200 molecules of hydrophilic ROOH per reaction center, but only four molecules of highly lipophilic ROOH. The limited formation of the latter was due to the limited supply of its precursor to the reaction, suggesting that it represented structurally fixed peroxides, i.e., either protein peroxides or peroxides of the lipids tightly bound to the core complex. These ROOH forms, likely including several species derived from lipid peroxides, may mediate the donor side-induced photoinhibition of PSII via protein modification.



Photosystem II (PSII) is a pigment–protein complex located in the thylakoid membrane as a homodimer. Recent crystallographic investigations of cyanobacterial PSII showed that the "core" complex of PSII contains at least 20 protein subunits, 35 chlorophyll (Chl) molecules, 12 molecules of carotenoids, and at least 14–20 integral lipid molecules per monomer.[1−3] PSII can be divided into two basic functional blocks. The first one is the photochemical reaction center (RC), where light energy absorbed by Chl is transformed into the energy of separated charges to form the oxidized primary electron donor, $P_{680}^{+\bullet}$, the strongest biological oxidant with a redox potential of 1.1–1.27 V.[4] The second one is the water-oxidizing complex (WOC) with the Mn_4CaO_5 cluster.[3] The WOC is oxidized via the sequential absorption of photons and charge separation in the RC. As a result, intermediate S states (S_0–S_4) are formed, and the transition from S_4 to S_0 is accompanied by the oxidation of two molecules of water and the formation of O_2.

PSII itself is the source of molecular oxygen, and at the same time, it can produce reactive oxygen species (ROS) under illumination[5] by three types of reactions as follows. Type I is the univalent reduction of O_2 to superoxide anion radical ($O_2^{-\bullet}$) on the acceptor side, where the generated reduction potential is sufficiently low (−600 mV) for the reaction. Possible donors of electrons to O_2 are the reduced forms of the primary electron acceptor, pheophytin ($Pheo^{-\bullet}$),[6] and the primary (Q_A^-) and secondary (Q_B^-) acceptor quinones,[7] and the plastosemiquinone that is produced via the proportionation between plastoquinone and plastoquinol.[8,9] There is also evidence that cytochrome b_{559} can reduce O_2.[10,11] $O_2^{-\bullet}$ is subsequently converted to H_2O_2 and O_2 via spontaneous or enzyme-catalyzed dismutation. Formation of H_2O_2 on the acceptor side was shown by a luminol-peroxidase assay.[6,7] From H_2O_2, hydroxyl radical ($HO^\bullet$) was formed via the Fenton mechanism.[12] Type II is the photosensitization effect of the reaction center P_{680} to form singlet oxygen ($^1O_2$). In the absence of electron acceptors, charge recombination in the primary ion radical pair of PSII ($P_{680}^{+\bullet}Pheo^{-\bullet}$) is facilitated and the triplet-state Chl, $^3P_{680}$*, is formed. $^1O_2$ is mainly generated through the interaction of $^3P_{680}$* with O_2.[13] These two mechanisms are physiologically relevant to acceptor side-induced photoinhibition of PSII.[14]

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Type III is the formation of ROS on the donor side. In PSII membranes with a partially impaired WOC, formation of H_2O_2 on the donor side has been reported.[7,15] The production mechanism is assumed to consist of divalent oxidation of two molecules of H_2O,[5] although the exact mechanism has yet to be elucidated. H_2O_2 may be further oxidized to $O_2^{-\bullet}$ or reductively cleaved to form $HO^{\bullet}$.[5] Under more severe conditions by which the WOC is totally inactivated, the oxidized primary ($P_{680}^{+\bullet}$) and secondary ($TyrZ^{\bullet}$) electron donors of PSII will stay a relatively long time, and hence, their chances of oxidizing surrounding molecules such as chlorophylls, carotenoids, and amino acids will increase.[16−19] This is relevant to the donor side-induced photoinhibition; the RC becomes labile to light when the supply of electrons from the WOC is stopped.[14,20] Because the addition of exogenous electron donors suppresses the photoinhibition in Mn-depleted PSII membranes,[21] the damage to PSII components is ascribed to the formation of $P_{680}^{+\bullet}$ and $TyrZ^{\bullet}$. Chen et al.[22] reported that, in Mn-depleted PSII membranes, $O_2^{-\bullet}$ formed on the acceptor side was involved in PSII photoinactivation but regarded ROS formation on the donor side unlikely. Thus far, it is unclear whether ROS and related species are formed in the absence of the Mn cluster and participate in the injury of RC components.

To characterize the formation of ROS in PSII, we have previously assessed the light-dependent O_2 consumption in PSII membranes. In the PSII membranes holding intact WOC, the O_2 consumption rate was very low [2 μmol (mg of Chl)$^{-1}$ h^{-1}], but in the alkaline-treated, Mn-depleted PSII membranes, the rate was 6-fold higher. O_2 consumption was found to be associated, at least partially, with the generation of a positive charge(s) on the donor side of PSII because it was inhibited by diuron and suppressed by PSII electron donors diphenylcarbazide (DPC) and ferrocyanide.[23] Further study revealed that multiple molecules of O_2 per reaction center were consumed on a single-turnover flash on the Mn-depleted PSII membranes.[24] On the basis of these results, we have proposed that the formation of hydroperoxides proceeds via a radical chain mechanism as follows:

$$Chl^{+\bullet}\ (\text{or } TyrZ^{\bullet}) + RH \rightarrow Chl(TyrZ) + R^{\bullet}$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

where RH, $R^{\bullet}$, $ROO^{\bullet}$, and ROOH represent an organic molecule, its radical, its peroxyl radical, and its peroxide, respectively. In this mechanism, organic peroxides such as lipid peroxides are formed, but H_2O_2 is not. Lipid peroxides are candidate species that are initially produced during the donor side-induced photoinhibition, because it has been recently found that lipid peroxide-derived aldehydes modify OEC33 and other PSII proteins during heat stress,[25] a situation leading to the donor side-induced photoinhibition.

In this study, we verified the hypothetical production of ROOH on the donor side of PSII. Using a lipophilic fluorescence probe 2-(4-diphenylphosphanylphenyl)-9-(1-hexylheptyl)anthra[2,1,9-*def*,6,5,10-*d*′*e*′*f*′]diisoquinoline-1,3,8,10-tetraone (Spy-HP) specific to peroxides,[26,27] we confirmed the light-dependent formation of peroxides in Mn-depleted PSII membranes. At least two types of ROOH, one lipophilic and the other hydrophilic, were distinguished, and both were insensitive to exogenously added catalase. The implications of this formation of peroxide in PSII photoinhibition are discussed.

MATERIALS AND METHODS

Oxygen-evolving PSII membranes prepared from spinach leaves[28] were suspended (2 mg of Chl/mL) in a medium containing 20 mM Mes-NaOH (pH 6.5), 35 mM NaCl, 0.33 M sucrose, and 10% glycerol and stored at −76 °C. They were washed once with a medium containing 50 mM Mes-NaOH (pH 6.5) and 35 mM NaCl (suspension medium). PSII membranes deprived of Mn were obtained by high-pH treatment as described previously.[29] The Chl concentration was determined in 80% acetone.[30]

The rate of light-dependent evolution and consumption of O_2 was measured using a Clark-type oxygen electrode for 60 s after the start of actinic illumination (λ > 650 nm; 1500 μmol of photons m^{-2} s^{-1}). The measurements were taken at 25 °C with 20 μg of Chl/mL in the suspension medium.

To identify the hydroperoxides photoproduced in PSII, the following approach was used. PSII membranes were suspended in the suspension medium at a concentration of 50 μg of Chl/mL and illuminated or kept in darkness at 20 °C, and then an aliquot (100 μL) was added to 2.7 μM Spy-HP (Dojindo Laboratories, Kumamoto, Japan) in ethanol (900 μL). After being incubated at 37 °C, the samples were centrifuged at 12000*g* for 2 min. The supernatant was collected, and its fluorescence spectrum (530−620 nm; λ_{ex} = 524 nm) was recorded with a Cary Eclipse spectrofluorimeter (Varian). The level of quenching of the Spy-HPOx fluorescence due to Chl was 10% at a concentration of 5 μg of Chl/mL (the concentration before PSII membranes were removed by centrifugation) and was negligible after centrifugation of the mixture. The difference between the fluorescence spectra of illuminated and unilluminated PSII membranes is designated the "light minus dark" fluorescence spectrum.

For determining the amounts of peroxides, standard data were calculated from the fluorescence intensity as follows. *m*-Chloroperbenzoic acid (MCPBA), as a model of a lipophilic hydroperoxide, at 0.5 μM caused an increase in the fluorescence of Spy-HPOx by 207 units in 5 min. H_2O_2 at 5 μM increased the fluorescence by 200 units in 180 min. *tert*-Butyl hydroperoxide (TBHP) at 5 μM increased the fluorescence by 85 units in 180 min.

RESULTS

Detection of Lipophilic and Hydrophilic Peroxides with Spy-HP. Light-induced formation of ROOH in PSII membranes was analyzed by the fluorescent probe Spy-HP. The probe is a derivative of diphenyl-1-pyrenylphosphine, a well-established fluorescent probe specific to peroxides. Because it has a bulky hydrophobic tail, Spy-HP has a high affinity for lipophilic hydroperoxides (LP-OOH), such as methyl linoleate hydroperoxide, and reacts very rapidly to form its oxidized product, Spy-HPOx, resulting in a significant increase in fluorescence.[26] Compared with the reaction with LP-OOH, its reaction with H_2O_2 and $HO^{\bullet}$ is very slow, and those with $O_2^{-\bullet}$, alkyl hydroperoxyl radical, nitric oxide, and peroxinitrite are negligible.[27] To obtain insight into the specificity of the probe for various peroxide species, we examined the reaction kinetics of Spy-HP with the following three peroxides: MCPBA, which is a model LP-OOH,[26] and TBHP and H_2O_2, both of which were included to represent water-soluble hydrophilic peroxides (HP-OOH) (Figure 1).

With 0.5 μM MCPBA, Spy-HP was oxidized within 5 min (Figure 1, curve 1) as previously reported.[26] Longer incubations of up to 180 min did not lead to a further increase or decrease in the fluorescence intensity. Thus, the reaction of MCPBA, an LP-OOH, with Spy-HP was completed within 5 min, and the oxidized product, Spy-HPOx, was stable for at least 180 min. This rapid reaction was also observed for methyl linoleate peroxide, another LP-OOH.[27] In contrast, with hydrophilic H_2O_2 or TBHP, even at a concentration (5 μM) 10-fold higher than that of MCPBA, the Spy-HP fluorescence did not increase in 5 min. However, with an increasing incubation time, a gradual increase in the fluorescence intensity was observed (Figure 1, curves 2 and 3). Thus, the reaction of HP-OOH with Spy-HP was very slow and was not completed after 180 min.

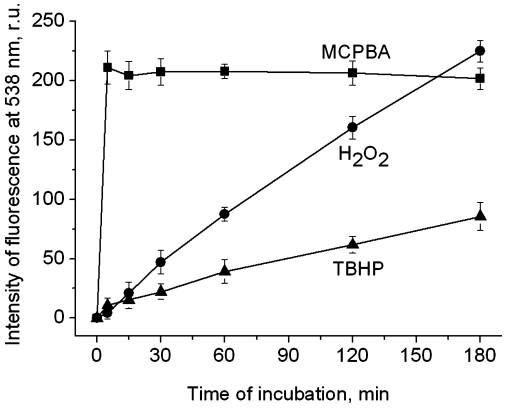

Figure 1. Time course of the Spy-HP oxidation with peroxides. MCPBA at 0.5 μM (■), H_2O_2 at 5 μM (●), or TBHP at 5 μM (▲) was added to the Spy-HP solution in ethanol, and the mixture was incubated at 37 °C for the indicated time. Spy-HP fluorescence in the absence of a peroxide was subtracted as background.

Investigation of the dependence of Spy-HP fluorescence on the TBHP concentration showed that the 5 min fluorescence signal was constant over the whole range of TBHP concentrations (1−10 μM), but the magnitude of the 180 min fluorescence signal increased with an increasing TBHP concentration (Figure 2). Thus, using Spy-HP, we can detect not only LP-OOH but also HP-OOH, although the sensitivity of Spy-HP to the latter is very low.

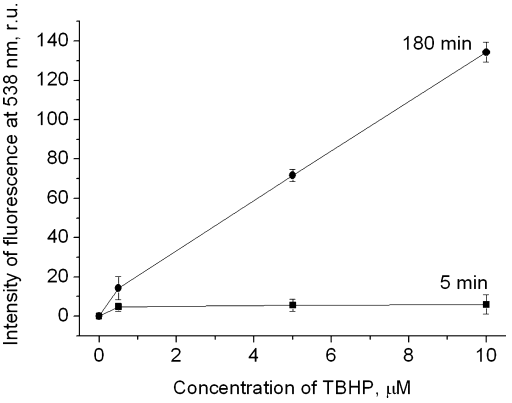

Figure 2. Dependence of the fluorescence intensity of Spy-HPOx on the concentration of TBHP. The Spy-HP solution was incubated in the presence of TBHP for 5 (■) and 180 min (●) at 37 °C. Spy-HP fluorescence in the absence of TBHP was subtracted as background.

On the basis of the differential reactivity of LP-OOH and HP-OOH with Spy-HP, we devised a simple method for distinguishing the two types of peroxides in a mixture. Specifically, a mixture of peroxides is added to the Spy-HP solution and the fluorescence is measured after incubation for 5 and 180 min. The 5 min signal reflects the fluorescence due to LP-OOH. The 180 min signal consists of both the fluorescence due to LP-OOH and that due to HP-OOH. The difference between the two signals, i.e., the 180 min signal minus the 5 min signal, will represent the fluorescence due to HP-OOH only. It was also possible to estimate the amount of LP-OOH. Because the reaction of Spy-HP with LP-OOH is accomplished within 5 min, the 5 min fluorescence intensity should be proportional to the amount of LP-OOH. Using MCBPA as a standard LP-OOH, we could determine the amount of LP-OOH formed in illuminated PSII membranes (described below). Estimation of the HP-OOH level will not be unequivocal because the reaction is not accomplished even in 180 min and the reaction rate depends on the peroxide species. As described below, we also calculated the amount of photoproduced HP-OOH under certain assumptions.

Illumination on Mn-Depleted PSII Membranes Results in Peroxide Formation. To test our hypothesis that Mn depletion creates a peroxide formation site(s) in PSII membranes, we compared the photoproduction of peroxides in Mn-depleted PSII membranes with that in untreated, WOC-compatible ones. PSII membranes were illuminated (λ > 650 nm; 1500 μmol of photons m^{-2} s^{-1}) or incubated in the dark, added to a Spy-HP solution in ethanol, and incubated at 37 °C.

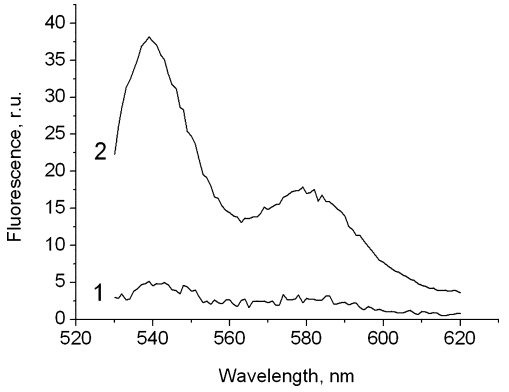

Figure 3. Light minus dark difference fluorescence spectra of Spy-HP obtained after a 5 min incubation of the illuminated untreated (1) and Mn-depleted (2) PSII membranes in the presence of the fluorescence probe. PSII membranes in the suspension medium (50 μg of Chl/mL) were illuminated (λ > 600 nm) with 1500 μmol of photons m^{-2} s^{-1} for 3 min.

The difference fluorescence spectra between the illuminated and unilluminated PSII membranes represented the light-induced formation of the peroxides (Figure 3). A 3 min illumination significantly increased the fluorescence intensity of Spy-HP in Mn-depleted PSII membranes (Figure 3, curve 2), while in WOC-compatible PSII, it led to just a small increase (8-fold lower) (Figure 3, curve 1). Thus, we confirmed that Mn-depleted PSII membranes created a peroxide formation site in PSII. This result corresponded with the finding that Mn

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depletion resulted in greater O_2 photoconsumption in PSII.[23] Hereafter, we use the fluorescence signal at 538 nm to determine the fluorescence increase.

Both Lipophilic and Hydrophilic Peroxides Are Photoproduced in Mn-Depleted PSII. To characterize the photoproduced peroxide species, the illuminated Mn-depleted PSII membranes were incubated for various lengths of time with Spy-HP, and the fluorescence increase was monitored [Figure 4 (○)]. Two components, fast and slow ones, were distinguishable: the fast component was the increase in the initial 5 min, and the slow one was the subsequent monotonic increase. On the basis of the finding that the rate of reaction of peroxides with Spy-HP depended on their hydrophobicity (Figure 1), the fast component was attributed to the formation of LP-OOH and the slow one to HP-OOH. This result indicated that at least two different kinds of peroxide were photoproduced in Mn-depleted PSII membranes.

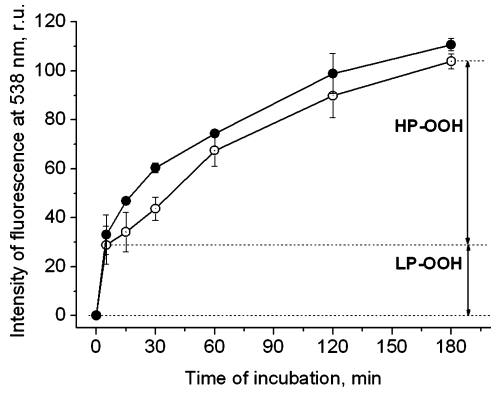

Figure 4. Determination of the time course of postillumination incubation of PSII membranes with Spy-HP allows the distinction between lipophilic and hydrophilic peroxides. Mn-depleted PSII membranes in the suspension medium (50 μg of Chl/mL) were illuminated (λ > 600 nm; 1500 μmol of photons m^{-2} s^{-1}) for 3 min in the absence (○) and presence (●) of SOD (200 units/mL) and catalase (500 units/mL) and then incubated with a Spy-HP solution in ethanol at 37 °C for the indicated amount of time.

It is known that PSII membranes can produce $O_2^{-\bullet}$ on the acceptor side and then H_2O_2 as a result of the disproportionation of $O_2^{-\bullet}$.[7] This raises the possibility that the photoproduced $O_2^{-\bullet}$ directly oxidizes Spy-HP to Spy-HPOx, although the rate of this reaction is very slow.[27] To examine whether $O_2^{-\bullet}$ and superoxide-derived H_2O_2 contributed to the HP-OOH fluorescence, we added superoxide dismutase (SOD) and catalase to the PSII membranes and illuminated them. As shown in Figure 4 (curve 2), the addition of these enzymes did not suppress the formation of LP-OOH and HP-OOH, indicating that neither $O_2^{-\bullet}$ nor H_2O_2 significantly contributed to the oxidation of Spy-HP. There was a slight stimulation of Spy-HPOx fluorescence by the addition of SOD and catalase. This might have been due to the disappearance of H_2O_2, which, had it been present, could have acted as a PSII donor (see below for details). To eliminate the possible influence of $O_2^{-\bullet}$ and H_2O_2, we performed all subsequent experiments in the presence of SOD and catalase.

Evidence of the Production of LP-OOH and HP-OOH on the Donor Side. The photoconsumption of O_2 in Mn-depleted PSII membranes was suppressed by the PSII electron donors DPC and ferrocyanide by nearly 70% and inhibited by DCMU.[24] In the same study, we also observed that the photoconsumption was slightly suppressed by the electron acceptor ferricyanide. We here examined the effects of these electron donors, the acceptor and the inhibitor, on the formation of ROOH in Mn-depeleted PSII membranes and compared them with those for O_2 photoconsumption (Table 1). The considerable (70−75%) suppression of the formation of LP-OOH and HP-OOH by 50 μM DCMU (Table 1) confirmed that this photoreaction was related with the electron flow in PSII. The electron acceptor ferricyanide decreased the level of photoproduction of both LP-OOH and HP-OOH by 10−20% (Figure 5). This effect of ferricyanide can be explained if we assume the production of a small amount of ROOH on the acceptor side, probably derived from the ROS produced in situ. Another explanation is that the photoreduced product ferrocyanide donated electrons and thereby suppressed the formation of ROOH on the donor side (described below). In either case, the result indicated that the ROOH formed on the acceptor side, if any, constitutes a minor fraction of the total amount of detected ROOH. When the electron donor DPC was added, the level of photoproduction of both LP-OOH and HP-OOH was decreased by 80−90%, as expected (Figure 5). This could be ascribed to the electron donor blocking the formation of the cation radical $P_{680}^{+\bullet}$ or $TyrZ^{\bullet}$ and thereby suppressing the production of organic peroxides. Another electron donor, ferrocyanide, showed a similar degree of suppression (Table 1). None of the added compounds affected the fluorescence yield of Spy-HPOx formed by LP-OOH and HP-OOH (data not shown).

To confirm that the electron donors suppressed the ROOH formation exclusively via their electron donation, we examined their effects on the stability of peroxides formed during illumination of Mn-depleted PSII preparations. The illuminated PSII samples were kept in the suspension medium in darkness at 20 °C for various lengths of time before they were mixed with Spy-HP (Figure 6). It was found that the peroxides spontaneously decomposed or were

Table 1. Effects of DCMU, Electron Donors, and Electron Acceptors on the Formation of LP-OOH and HP-OOH[a]

	fluorescence (relative units)		
	LP-OOH	HP-OOH	photoconsumption of O_2 [μmol (mg of Chl)$^{-1}$ h^{-1}][b]
no addition	39 ± 3.5 (100%)	84 ± 4.4 (100%)	11.5 ± 1.4 (100%)
1 mM $K_3[Fe(CN)_6]$	36 ± 3.8 (92%)	76 ± 6 (90%)	8 ± 0.2 (69%)
0.5 mM $K_4[Fe(CN)_6]$	12 ± 2.4 (31%)	47 ± 3.2 (56%)	3.5 ± 0.3 (30%)
0.25 mM DPC	3.5 ± 2.8 (9%)	24.5 ± 4.2 (29%)	5 ± 0.7 (43%)
50 μM DCMU	10 ± 2.1 (26%)	23 ± 3 (27%)	2 ± 0.2 (17%)

[a]None of the added compounds affected the fluorescence yield of Spy-HPOx formed by LP-OOH and HP-OOH. The PSII membranes in the suspension medium (50 μg of Chl/mL) were illuminated (λ > 650 nm) with 1500 μmol of photons m^{-2} s^{-1} for 3 min. [b]Data from ref 24.

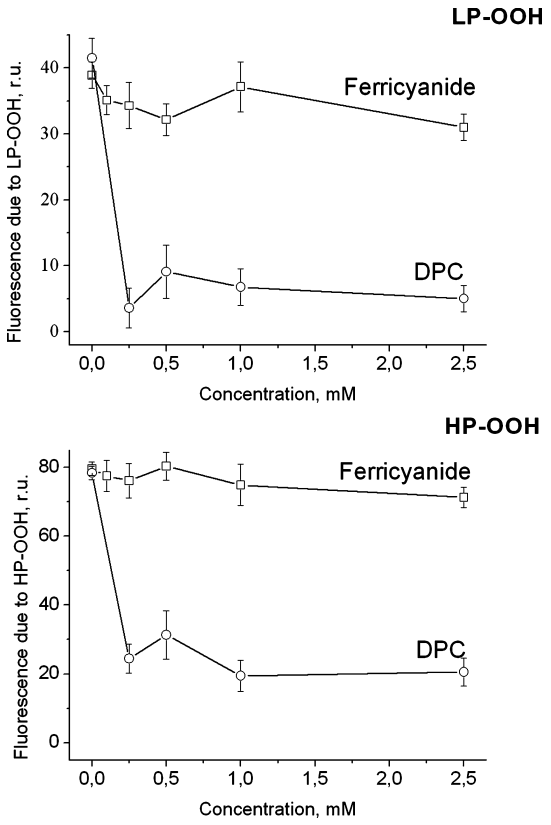

Figure 5. Effects of the electron acceptor ferricyanide (□) and the electron donor DPC (○) on the photoproduction of LP-OOH (top) and HP-OOH (bottom) in Mn-depleted PSII membranes. The PSII membranes in the suspension medium (50 μg of Chl/mL) were illuminated (λ > 650 nm) with 1500 μmol of photons m^{-2} s^{-1} for 3 min. The fluorescence levels due to LP-OOH and HP-OOH were determined as described in the legend of Figure 4.

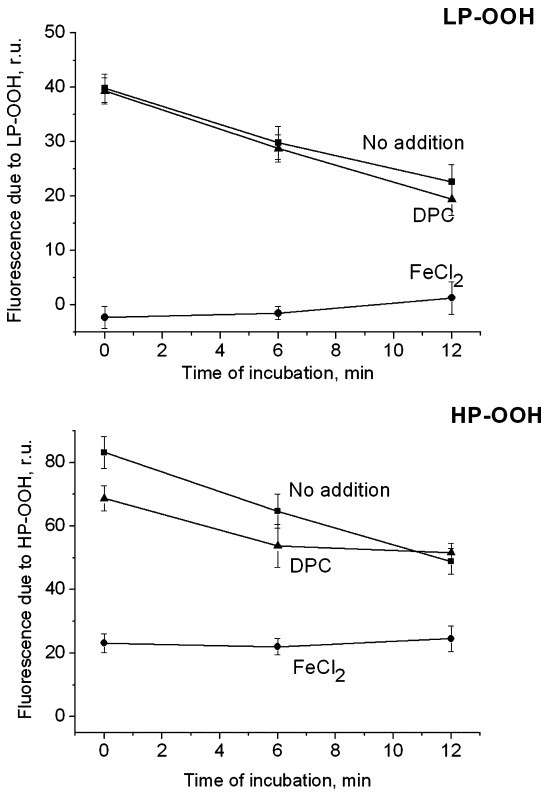

Figure 6. Effects of DPC and $FeCl_2$ on the stability of LP-OOH (top) and HP-OOH (bottom) formed in the illuminated PSII membranes. Mn-depleted PSII membranes were illuminated for 3 min with SOD and catalase as in Figure 4 and then incubated in the same medium at 20 °C without any addition (■) or with 1 mM DPC (▲) or 1 mM $FeCl_2$ (●) for the indicated amounts of time. The levels of fluorescence due to LP-OOH and HP-OOH were determined as described in the legend of Figure 4.

scavenged in the suspension medium: approximately 12 min was required for a 50% decrease. The half-lives of LP-OOH and HP-OOH were not affected by either 0.25 mM DPC or 1 mM ferrocyanide (the latter data are not shown). Thus, the suppression of formation of ROOH by DPC and ferrocyanide was ascribed to their donation of electrons to PSII. These results using Spy-HP were thus in good correspondence with the O_2 photoconsumption data and support our hypothesis that the photoconsumption of O_2 on the PSII donor side leads to the formation of ROOH via the radical chain reactions.

We further examined the effect of $FeCl_2$, a Fenton reagent that induces the decomposition of peroxides, on Spy-HPOx fluorescence (Figure 6). Postillumination addition of $FeCl_2$ to the Mn-depleted PSII membranes almost completely suppressed the oxidation of Spy-HP, while it did not quench the fluorescence of Spy-HPOx, which had been produced via the oxidation of Spy-HP with 0.5 μM MCPBA (data not shown). Thus, it was confirmed that the detected oxidants that were formed in Mn-depleted PSII membranes were peroxides. These results provide direct evidence of the photoproduction of two types of ROOH, LP-OOH and HP-OOH, on the donor side of PSII.

Estimation of the Yields of LP-OOH and HP-OOH. To determine the yields of LP-OOH and HP-OOH in Mn-depeleted PSII membranes, we obtained the light intensity dependence of the photoproduction of both types of ROOH. Mn-depleted PSII membranes were illuminated in the presence of SOD and catalase for 3 min at various light intensities, and

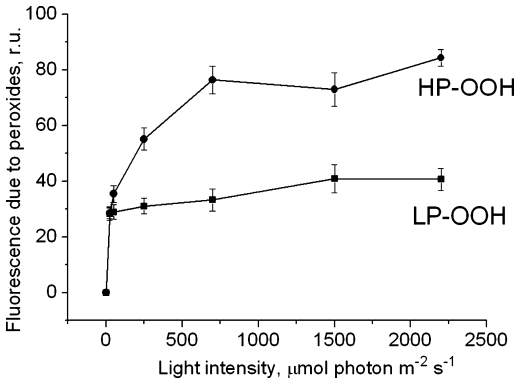

Figure 7. Dependence of the formation of LP-OOH (■) and HP-OOH (●) on light intensity. Mn-depleted PSII membranes were illuminated in the presence of SOD and catalase at various light intensities for 3 min. The levels of fluorescence due to LP-OOH and HP-OOH were determined as described in the legend of Figure 4.

then the Spy-HPOx fluorescence due to LP-OOH and HP-OOH was determined (Figure 7). LP-OOH and HP-OOH showed different light saturation curves; the former saturated at or below 25 μmol of photons m^{-2} s^{-1} and the latter

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at 750 μmol of photons m^{-2} s^{-1}. This difference was probably due to the rate of supply of the organic molecules to be oxidized (see Discussion).

From the fluorescence intensity, we estimated the amounts of peroxides as follows (for details, see Materials and Methods). (1) The amount of LP-OOH was determined from the 5 min fluorescence using MCPBA as the standard. (2) The amount of HP-OOH was determined from the "180 min minus 5 min" fluorescence using TBHP as a standard. At a light intensity of 25 μmol of photons m^{-2} s^{-1} for 3 min, the LP-OOH concentration reached 85 nM. On the basis of the Chl concentration of the sample (5 μg/mL) during the incubation with Spy-HP and the Chl/reaction center molar ratio of 250, the yield of LP-OOH was determined to be four molecules per reaction center. Similarly, the concentration of HP-OOH reached 1.8 and 4.5 μM (as TBHP equivalent) at 25 and 750 μmol of photons m^{-2} s^{-1}, respectively, in 3 min, corresponding to HP-OOH yields of 80 and 200 molecules per reaction center, respectively.

From the data at 25 μmol of photons m^{-2} s^{-1}, the rates of production of LP-OOH and HP-OOH in the initial 3 min illumination are estimated to be 0.34 and 7.2 μmol (mg of Chl)$^{-1}$ h^{-1}, respectively. At 750 μmol of photons m^{-2} s^{-1}, they reach 0.37 and 18 μmol (mg of Chl)$^{-1}$ h^{-1}, respectively. These values were comparable to the O_2 photoconsumption rate obtained previously [11.5 μmol (mg of Chl)$^{-1}$ h^{-1} [24]].

DISCUSSION

Organic Peroxides Are Formed on the Donor Side of PSII. In this study, using the peroxide-specific fluorescence probe Spy-HP, we demonstrated that electron transport-dependent formation of ROOH occurs on the donor side of PSII, which lacks the Mn cluster, based on the following results. (1) The identity of the detected chemical species as a peroxide(s) was assured by the fact that Fe^{2+}, a Fenton reagent, effectively suppressed the fluorescence increase (Figure 6). Furthermore, the failure of catalase to suppress the formation of peroxides (Figure 4) indicated that they were not H_2O_2, but rather ROOH. (2) The level of formation of peroxide in PSII was increased upon illumination (Figure 3) and inhibited by DCMU (Table 1), indicating that it depends on the electron transport in PSII. (3) In PSII, peroxide formation was possible both on the acceptor side and on the donor side (Figure 5). The major portion of the detected peroxides was attributed to those formed on the donor side, because the level of formation of peroxide was much larger in the Mn-depleted PSII membranes than in the WOC-compatible ones and was suppressed by the addition of electron donors (Figure 5 and Table 1).

The results described above correspond well with our previous results about the light-dependent O_2 consumption on the donor side of Mn-depleted PSII membranes,[23,24] with regard to the effects of electron donors and DCMU, and the reaction rate. We conclude that the peroxides are formed by the mechanism proposed in the introductory section. Specifically, the strong oxidizing power accumulated on the donor side oxidizes the surrounding organic molecules to their radicals. These react with O_2, and the resulting organic peroxyl radicals ($ROO^{\bullet}$) further oxidize surrounding molecules, thereby becoming ROOH.

There has been no report, to the best of our knowledge, of the formation of ROOH in illuminated PSII membranes. This is probably because the steady-state concentrations of ROOH are very low because of the instability of ROOH. The use of the highly sensitive probe Spy-HP was the key to detecting these peroxides.

Lipophilic Peroxides and Hydrophilic Peroxides. On the basis of the reactivity of various peroxides with Spy-HP, we could distinguish two types of peroxides, i.e., LP-OOH and HP-OOH, formed on the donor side of PSII (Figure 4). Each type of peroxide accumulated to different levels; in the 3 min illumination at a saturating intensity, LP-OOH accumulated to a level of four molecules per reaction center, while the level of HP-OOH reached 200 molecules per reaction center (Figure 7). Interestingly, the photoproduction of LP-OOH saturated with relatively weak light, 25 μmol of photons m^{-2} s^{-1}. This indicates that the availability of precursor molecules of this type of peroxide limits the peroxide formation even in weak light. In contrast, the formation of HP-OOH did not saturate up to 200 μmol of photons m^{-2} s^{-1}, indicating that the precursors of HP-OOH were supplied to the oxidation site much more quickly than the LP-OOH precursors.

Taking these characteristics into account, we presume the chemical identity of the respective types of peroxides based on the following rationale. LP-OOH would be the peroxides on the proteins in PSII, such as the D1 protein. Tyr radical and other radicals on the amino acid residues on a protein can react with O_2 to form the peroxyl radicals, and then they will oxidize neighboring molecules to form hydroperoxides.[31] Radicals formed by the oxidation of carotenoids[17,18] could also be a source of LP-OOH formation. Another candidate for LP-OOH is the peroxide of lipids that are tightly bound to the PSII core complex. In the PSII core complex from the cyanobacterium *Thermococcus elongatus*, 25 lipid molecules per PSII monomer are assigned, among which monogalactosyl-diacylglycerol and digalactosyl-diacylglycerol are major components.[1,2] Similar lipid compositions are expected for higher plants, including spinach.[32] These thylakoid lipids, containing unsaturated fatty acids such as oleic acid as major constituents,[32] are readily oxidized to the peroxides. Because these possible precursors of peroxides, either proteins or bound lipids, are structurally fixed in the core complex, their supply to the oxidation reaction would be limited. This could be the reason for the limited number of LP-OOH molecules formed.

The precursor to HP-OOH, in contrast, should be abundant in the PSII membranes. In this respect, membrane lipids appear to be the only possible candidates. On the other hand, on the basis of their poor reactivity with Spy-HP (Figure 4), the resulting HP-OOH molecules should be more polar (and probably smaller) than simple lipid peroxides such as methyl linoleate peroxide.[27] One candidate species for satisfying these conditions is the peroxides derived from the degradation products of lipid peroxides. When polyunsaturated fatty acids such as linoleic and linolenic acids react with radicals, hydrogen at the central carbon of their pentadienyl structure is abstracted to form their radicals, which quickly react with O_2 to form the fatty acid peroxides. Lipid peroxides can enzymatically or spontaneously decompose to secondary metabolites via three main routes: (i) homolytic scission of the C−C bonds via lipid alkoxyl radical or by cleavage with hydroperoxide lyase, (ii) rearrangement and consecutive oxidation of the monohydroperoxides, and (iii) polymerization reaction leading to intermolecular cross-links between lipid peroxides.[33] The homolytic scission of lipid hydroperoxides leads to the formation of a variety of carbonyl species and oxo fatty acids, including those with unsaturated bonds, such as *cis*-3-nonenal

and *cis*-3-hexenal.[34,35] These *cis*-3-alkenals are further oxidized to form radicals and then peroxides.[36] Oxidation of thylakoid lipids to their peroxides has been suggested by the detection of various aldehydes, end products of the peroxides, in stressed leaves.[25,37,38] It is possible that the oxidants accumulated on the PSII donor side also oxidize the membrane lipids and their descendant *cis*-3-alkenals.

Physiological Relevance of the Peroxides Formed on the Donor Side of PSII. Donor side-induced photoinhibition starts with the inactivation of the WOC. This can be observed under heat stress,[39] UV-B irradiation,[40,41] and visible light even at moderate intensity.[20,42,43] Illumination of the WOC-disabled PSII will lead to the enzymic degradation of D1 protein. It is generally accepted that specific proteases will degrade "damaged" D1 proteins. For example, the protease FtsH2 in *Synechocystis* 6803 plays an important role in the rapid degradation of D1 protein under the conditions leading to the donor side-induced photoinhibition, such as UV-B damage, and illumination of cells in the presence of ammonia or in the absence of extrinsic proteins PsbO and PsbV (ref 44 and references cited therein). However, there is little information about the chemistry of the damage itself. The formation of LP-OOH suggests the occurrence of peroxides on the D1 protein, and this type of modification can be an initial event to trigger protein degradation in the donor side-induced photoinhibition. HP-OOH also can mediate protein modification indirectly. Small reactive aldehydes such as malondialdehyde and acrolein, derived from lipid peroxides or HP-OOH, have been shown to occur and to modify several PSII proteins in vivo under heat stress conditions.[25] Although the levels of accumulation of LP-OOH and HP-OOH are very low and their chemical identity has yet to be elucidated, the formation of these potentially reactive species should be significantly important in the photoinhibition mechanisms.

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ABBREVIATIONS

Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; HP-OOH, hydrophilic hydroperoxides; LP-OOH, lipophilic hydroperoxides; MCPBA, *m*-chloroperbenzoic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; P_{680}, primary electron donor of PSII; Pheo, pheophytin, the primary electron acceptor of PSII; PSII, photosystem II; Q_A, primary plastoquinone electron acceptor of PSII; Q_B, secondary plastoquinone electron acceptor of PSII; RC, reaction center; ROOH, organic peroxides; Spy-HP, 2-(4-diphenylphosphanylphenyl)-9-(1-hexylheptyl)anthra[2,1,9-*def*,6,5,10-*d'e'f'*]diisoquinoline-1,3,8,10-tetraone; TBHP, *tert*-butyl hydroperoxide; TyrZ, redox active tyrosine residue of D1 protein; WOC, water-oxidizing complex.

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