Improvement by Benzoquinones of the Quantum Yield of Photoactivation of Photosynthetic Oxygen Evolution: Direct Evidence for the Two-Quantum Mechanism†

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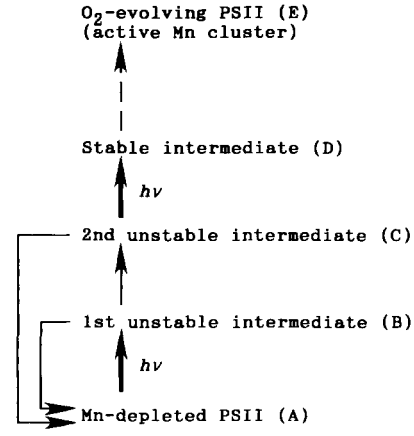
ABSTRACT: Effects of eight differently substituted 1,4-benzoquinones (BQs) on the quantum yield of photoactivation of oxygen evolution (reconstitution of the Mn cluster) were examined with wheat photosystem II (PSII) membranes depleted of the Mn cluster by treatment with 1.0 mM NH_2OH. Illumination with 10 flashes at 0.25-s intervals of the PSII membranes in the presence of 2.0 mM Mn^{2+}, 20 mM Ca^{2+}, and 1.2 M Cl^- restored 14% of oxygen-evolving activity destroyed by the NH_2OH treatment. Among the benzoquinones tested, DBMIB (2,5-dibromo-3-methyl-6-isopropyl-BQ) and tetramethyl-BQ did not enhance the activity recovery, but all the others doubled the recovery when present at their optimal concentrations during illumination. The order of effectiveness was tetrabromo-, phenyl-, and 2,6-dichloro-BQs ≥ 2,5-dichloro-BQ > tetrachloro-BQ > 2,5-dimethyl-BQ, though the differences were small. This order reflects their efficiencies as electron acceptors of PSII. This finding, together with others, suggests that the enhancement of activity recovery results from rapid oxidation by the benzoquinones of the reduced form of the quinone acceptors in PSII, Q_A^- and Q_B^-, which cause loss of an oxidized intermediate through charge-recombination reaction with Mn^{3+}. The flash-number dependence of the recovery of oxygen-evolving activity indicated that the activity was not restored after one flash but recovered significantly after illumination with two flashes and then further increased upon additional flashes. This provides direct evidence that the minimum quantum requirement for photoactivation is two.

Photosynthetic oxygen evolution is catalyzed by a Mn cluster that is bound to an intrinsic part of the photosystem II (PSII)[1] complex at the lumenal surface of thylakoid membrane (Miyao & Murata, 1987). The Mn cluster consists of four Mn atoms of an average valence higher than 2, and its structure and functioning mechanism have been the subject of much research [for reviews, see Pecoraro (1988) and Brudvig et al. (1989)].

Photoactivation of oxygen evolution is a process by which an active Mn cluster is formed in the PSII complex under illumination [for a review, see Radmer and Cheniae (1977)]. This process involves oxidation of Mn^{2+} by photochemical reaction in PSII and subsequent assembly of the oxidized Mn to the functional Mn cluster (Radmer & Cheniae, 1977). Kinetic analyses of the photoactivation have proposed that the process proceeds via at least two photoreactions and a dark reaction in between (Radmer & Cheniae, 1971, 1977; Inoue et al., 1975; Ono & Inoue, 1987) as shown in Scheme I. The PSII complex depleted of the Mn cluster and inactive in oxygen evolution (A) is converted to an intermediate (B) by the first photoreaction. This intermediate is unstable and decays back to A unless it is successfully rearranged in darkness to another intermediate (C). The second intermediate is also unstable and decays back to A unless the next photoreaction takes place. The second photoreaction converts the second unstable intermediate (C) to a stable intermediate (D) which is finally converted to a functional Mn cluster (E) in darkness. Both photoreactions (conversions of A to B and of C to D) have been proposed to involve photooxidation of Mn^{2+} to yield Mn^{3+} (Radmer & Cheniae, 1971; Ono & Inoue, 1987; Tamura & Cheniae, 1987a; Miller & Brudvig, 1990).

Scheme I



Understanding of the mechanism of photoactivation is of a great interest not only to clarify how the PSII complex acquires oxygen-evolving capacity in the course of chloroplast development but also to obtain an insight into the structure and binding site of the Mn cluster. In addition, it is expected to offer a technique for replacing some of the Mn atoms in the cluster by other metals, thereby providing information as

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[1] Abbreviations: BQ, 1,4-benzoquinone; Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; PSII, photosystem II; Q_A, primary quinone acceptor of photosystem II; Q_B, secondary quinone acceptor of photosystem II; Y_D, tyrosine residue in photosystem II that gives rise to EPR signal II_{slow} when oxidized.

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to the chemistry of the cluster that has not so far been obtained through usual studies of the native oxygen-evolving Mn cluster. Unfortunately, however, analysis of the photoactivation process has not made much progress yet, because of a very low quantum yield of the process of an order of 0.01 (Cheniae & Martin, 1971).

This low quantum yield has been ascribed to a low yield of the second unstable intermediate (C), that is, inefficient conversion from A to C (Tamura & Cheniae, 1987a). Recently, Miller and Brudvig (1990) have suggested that photooxidation of Mn^{2+} in the first photoreaction occurs at a very low quantum efficiency and proposed that this step limits the overall yield of photoactivation.

Experimentally, the quantum yield of photoactivation depends on not only Mn^{2+} concentration but also concentrations of Ca^{2+} and Cl^- during illumination. Calcium ion is essential for the reconstituted Mn clusters to evolve oxygen (Tamura et al., 1989) but also acts as a competitive inhibitor for Mn^{2+} binding when present during illumination (Ono & Inoue, 1983; Miller & Brudvig, 1989). Chloride ion enhances the photoactivation yield by suppressing the decay of the second unstable intermediate and by stabilizing the reconstituted Mn clusters and also modulates Mn^{2+} requirement of photoactivation (Miyao & Inoue, 1991b). Thus, the ratio of these ions is an important factor for the photoactivation yield. In PSII membranes depleted of the plastoquinone pool and photosystem I, an artificial electron acceptor of PSII is additionally required for the photoactivation, and DCIP has been reported to be most suitable when the photoactivation is effected by continuous light (Tamura & Cheniae, 1987a; Miyao & Inoue, 1991a). When the concentrations of those ions and DCIP were optimized, more than 80% of oxygen-evolving activity could be restored under illumination with continuous light (Miyao & Inoue, 1991a,b). However, the quantum yield of photoactivation remained still lower than 0.01 [see Miyao and Inoue (1991b)].

Here, we report that the quantum yield of photoactivation was increased to 0.1–0.2 by use of 1,4-benzoquinones, efficient electron acceptors of PSII. We also present experimental evidence for the previously proposed model that two successive photoreactions in PSII are sufficient to reconstitute the Mn cluster.

Experimental Procedures

Wheat PSII membranes prepared from thylakoids with Triton X-100 were first treated with 1.5 M NaCl in darkness, to remove extrinsic 23-kDa and 16-kDa proteins from the PSII complex, and then further treated with 1.0 mM NH_2OH in darkness to remove Mn atoms from the complex (Miyao & Inoue, 1991a). Handling of the treated membranes were done under dim green light. A mixture of the 23-kDa and 16-kDa proteins was prepared as described previously (Miyao & Inoue, 1991a).

Conditions for photoactivation of oxygen evolution were essentially the same as those reported previously (Miyao & Inoue, 1991a,b). NH_2OH-treated PSII membranes which had been kept in darkness for more than 2 h were suspended at 0.25 mg of Chl/mL in 1.2 M Cl^-, 0.4 M sucrose, and 25 mM Mes-NaOH (pH 6.5) containing designated concentrations of Mn^{2+}, Ca^{2+}, and an artificial electron acceptor. Mn^{2+} and Ca^{2+} were supplied as chlorides, and Cl^- concentration was adjusted to 1.2 M with NaCl. The electron acceptor was taken from a stock solution prepared in dimethyl sulfoxide, except for DCIP, which was dissolved in 10 mM Mes-NaOH (pH 6.5). The final concentration of the solvent was 4% (v/v) in all cases. The membrane suspension was incubated in darkness for 1–2 min at 25 °C and illuminated with flashing light from a 4-μs xenon flash lamp at 25 °C. When Ca^{2+} was absent during illumination, the suspension was supplemented with designated concentrations of Ca^{2+} by adding 2.0 M $CaCl_2$ containing 0.4 M sucrose and 25 mM Mes-NaOH (pH 6.5) immediately after the illumination. After being kept in darkness for 15 min at 25 °C, the suspension was diluted with 0.4 M sucrose and 25 mM Mes-NaOH (pH 6.5) containing 23-kDa and 16-kDa proteins, and oxygen-evolving activity was measured at 25 °C with 0.8 mM phenyl-BQ using a Clark-type oxygen electrode. Activity recovery was expressed by the oxygen-evolving activity of photoactivated membranes as percentage of that of the NaCl-treated membranes which were similarly treated with Mn^{2+}, Ca^{2+}, and Cl^- but in darkness. The activities of NaCl-treated membranes were equivalent to those of untreated PSII membranes.

Substituted 1,4-benzoquinones were obtained commercially in a special grade as pure as possible and used without further purification except for phenyl-BQ, which was purified according to Yocum (1980). Chl concentration was determined according to Arnon (1949).

Results

Effects of Benzoquinones on Photoactivation Yield. To compare the effectiveness of various artificial electron acceptors in enhancing the quantum yield of photoactivation, we employed an ion condition, 2.0 mM Mn^{2+}, 20 mM Ca^{2+}, and 1.2 M Cl^-, that gave the maximum recovery among those tested. We examined nine electron acceptors, DCIP and eight differently substituted 1,4-benzoquinones. Recoveries of oxygen-evolving activity of NH_2OH-treated PSII membranes by illumination with 10 flashes at 0.25-s intervals were compared at a wide range of acceptor concentrations. With most acceptors tested, the recovery increased with acceptor concentration to reach the maximum and then decline (data not shown). The recovery decline at higher acceptor concentrations is ascribed to an inhibitory action of reduced acceptors formed during illumination (Miyao & Inoue, 1991a) and/or inhibition by added acceptors of oxygen-evolving activity supported by phenyl-BQ (not shown).

The maximum recovery attained with each acceptor and its optimum concentration giving the maximum are presented in Table I. Here, the acceptors are listed in the order of effectiveness. Without added acceptor, 14% of the activity lost by NH_2OH treatment was restored by 10-flash illumination. Among the benzoquinones tested, DBMIB and tetramethyl-BQ did not at all improve the recovery, while all the others doubled the recovery at their respective optimum concentrations. Tetrabromo-, phenyl-, and 2,6-dichloro-BQs were most effective and the recovery reached 30% of the original. DCIP also improved the recovery but to a lesser extent.

Table I also lists the K_m and V_{max} (maximum activity) values of each benzoquinone in supporting electron transport in PSII membranes retaining an intact Mn cluster. These parameters could not be obtained for tetrabromo- and tetrachloro-BQs because of their low solubility in aqueous suspensions. However, they are deduced to be close to those of phenyl- and dichloro-BQs, since tetrabromo- and tetrachloro-BQs supported the activity as efficiently as phenyl- or dichloro-BQ when their concentrations were lower than 30 μM (data not shown). Thus, it is likely that the optimum concentration for photoactivation well reflects K_m for electron-transport activity. As for the extent of activity recovery, the benzoquinones that exhibit higher V_{max} appear to more effectively increase the recovery, though the correlation is not so strict. Since V_{max} reflects the rate of electron transfer from the acceptor side of

Table I: Effectiveness of Various Artificial Electron Acceptors in Supporting Photoactivation of Oxygen Evolution in Mn-Depleted PSII Membranes and Electron Transport in Oxygen-Evolving PSII Membranes[a]

	photoactivation		electron transport		
electron acceptors	optimum conc. (μM)	activity recovery [μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}]	K_m (μM)	V_{max} [μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}]	$E_{1/2}$ (mV)
tetrabromo-BQ	5	210 (30%)	*	*	+106
phenyl-BQ	20	210 (30%)	80	780	−390
2,6-dichloro-BQ	20	210 (30%)	90	600	−30
2,5-dichloro-BQ	10	200 (29%)	70	630	−65
tetrachloro-BQ	5	190 (27%)	*	*	+140
2,5-dimethyl-BQ	1000	180 (26%)	1000	690	−557
DCIP	2.5	140 (20%)			
DBMIB	1–8	100 (14%)	(1–100)	(150–200)	−249
tetramethyl-BQ	100	100 (14%)	360	460	−738
none		100 (14%)			

[a] Photoactivation was effected by illuminating NH_2OH-treated PSII membranes with 10 flashes at 0.25-s intervals in the presence of 2.0 mM Mn^{2+}, 20 mM Ca^{2+}, 1.2 M Cl^-, and various concentrations of an electron acceptor. The illuminated membranes were diluted 25-fold with a medium containing the 23-kDa and 16-kDa proteins, and oxygen-evolving activity was measured in the presence of 0.8 mM phenyl-BQ at 10 μg of Chl/mL. Activity recovery represents the difference in oxygen-evolving activity before and after the flash illumination and is also expressed as percentage of the activity of NaCl-treated membranes in parentheses. The activities of NaCl-treated membranes and NH_2OH-treated membranes before illumination were 700 and 20 μmol of O_2·(mg of Chl)$^{-1}$·h^{-1}, respectively. Maximum recoveries with the acceptors at their respective optimum concentrations were presented. Effectiveness of the acceptors in supporting electron transport was examined by measuring oxygen-evolving activity of NaCl-treated PSII membranes in the presence of 10 mM $CaCl_2$ at various acceptor concentrations. The K_m value and maximum activity (V_{max}) were obtained from the results by the Lineweaver–Burk plot. For tetrabromo- and tetrachloro-BQs these parameters could not be determined because of their low solubility in aqueous suspensions [marked with an asterisk (*)]. With DBMIB, the activity was almost constant at concentrations tested (1—100 μM). $E_{1/2}$ represents polarographic half-wave potentials determined in dimethylformamide by Prince et al. (1982).

PSII (predominantly from Q_B^{2-}) to added benzoquinones (Tanaka-Kitatani et al., 1990), it is inferred that the benzoquinones that are efficient electron acceptors are effective in photoactivation as well. The effectiveness of benzoquinones in photoactivation, on the other hand, did not correlate with $E_{1/2}$, the approximate oxidation–reduction midpoint potential (see Prince et al., 1982).

Figure 1 presents the dependences on flash interval of activity recovery by 10 flashes with various electron acceptors. With no acceptor added, the dependence gave a broad bell-shaped curve exhibiting the maximum recovery at an interval of 0.25–0.5 s. DCIP slightly increased the recovery but did not affect the overall shape of the dependence curve. All benzoquinones remarkably increased the recovery at every interval longer than 0.01 s, but the shapes of resultant dependence curves were different from each other. With 2,6-dichloro-BQ, the recovery steeply increased with flash interval up to 0.25 s and then declined, giving a typical bell-shaped dependence on flash interval. A similar bell-shaped dependence was obtained with 2,5-dichloro- and tetrachloro-BQs (data not shown). With phenyl-BQ or tetrabromo-BQ, in contrast, the recovery increased with flash interval in the same manner as with 2,6-dichloro-BQ, but the decline at longer intervals was much suppressed. According to the model of photoactivation as depicted in Scheme I, the flash-interval dependence reflects the relative yield of the second unstable intermediate (C); rise and decay of the bell-shaped curve correspond to formation of the intermediate (conversion from A to C) and its decay in darkness (conversion of C to A), respectively. It is thus concluded that all benzoquinones at their respective optimum concentrations equally facilitate the formation of the second unstable intermediate and that phenyl- and tetrabromo-BQs additionally act to suppress the decay of this intermediate.

Another finding in Figure 1 is that none of the artificial electron acceptors tested increased the recovery when the flash interval was 0.01 s. This contrasts with the effects of increase in Mn^{2+} concentration, which uniformly enhanced the recovery at all intervals ranging from 0.01 to 8 s (Miyao & Inoue, 1991b). At flash intervals as short as 0.01 s, the activity recovery is determined mainly by the efficiency of rapid processes (the overall yield of two photoreactions on the quantum basis) but does not significantly depend on rates of slow processes which proceed in a time scale longer than the flash interval (e.g., conversion of B to C). This implies that the recovery at the flash interval of 0.01 s is an indirect measure of the quantum yield of photoreactions. We may thus conclude that the quantum yield of photoreactions is not improved by the artificial electron acceptors but will be determined by Mn^{2+} concentration.

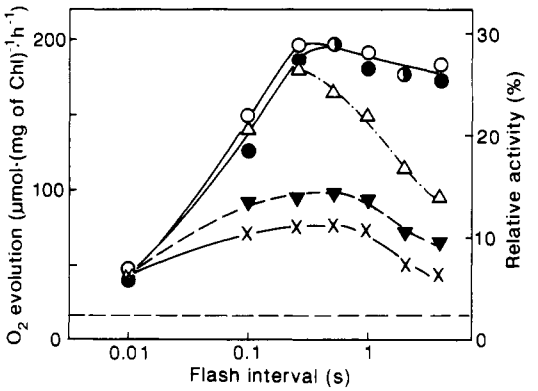

FIGURE 1: Effects of artificial electron acceptors on flash-interval dependence of photoactivation with flashing light. NH_2OH-treated PSII membranes were illuminated with 10 flashes at designated intervals in the presence of 2 mM Mn^{2+}, 20 mM Ca^{2+}, 1.2 M Cl^-, and designated electron acceptors. The oxygen-evolving activity was measured as described in the footnote to Table I. Averages of three measurements were presented. 100% represents oxygen-evolving activity of NaCl-treated PSII membranes, and the horizontal broken line represents the activity before illumination. (×) No acceptor added; (▼) 2.5 μM DCIP; (△) 20 μM 2,6-dichloro-BQ; (●) 5 μM tetrabromo-BQ; (○) 20 μM phenyl-BQ.

In the above experiments, we compared the activity recovered by illumination with 10 flashes and found the conditions for a high flash yield of photoactivation. However, these conditions were not always suitable to fully reactivate oxygen evolution. Figure 2 shows the changes in activity recovery with 20 μM phenyl-BQ upon increasing the flash number. When the flash interval was 0.01 s, oxygen-evolving activity gradually increased with flash number to reach a maximum plateau level of about 25% of the original after illumination with 100 flashes. At flash intervals longer than

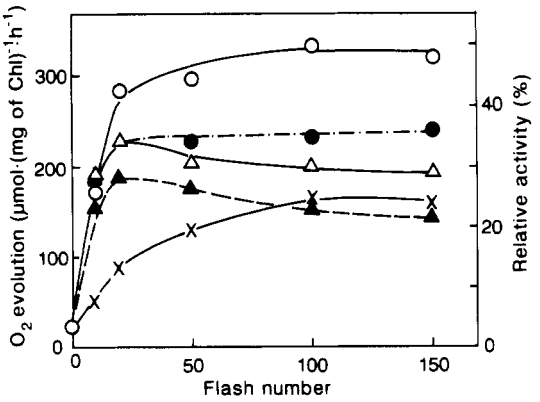

FIGURE 2: Changes in activity recovery on increasing flash number. NH_2OH-treated PSII membranes were suspended in 2.0 mM Mn^{2+}, 20 mM Ca^{2+}, 1.2 M Cl^-, and 20 μM phenyl-BQ, and illuminated with designated numbers of flashes at designated intervals. Other experimental conditions are the same as those in Figure 1. Flash interval: (×) 0.01 s; (▲) 0.1 s; (△) 0.5 s; (●) 1.0 s; (○) 4.0 s.

0.1 s, in contrast, the activity steeply increased with flash number, but this increase was suddenly terminated after about 20 flashes, and consequently, the maximum recovery levels remained around 30–50% of the original. Probably this results from a limited capacity of electron transfer from the quinone acceptors of PSII due to a relatively low concentration of phenyl-BQ. Assuming a molar ratio of 220 Chl per reaction center (Murata et al., 1984), 20 μM phenyl-BQ present in the photoactivation mixture corresponds to only 16 molecules of the benzoquinone per center. Even if all benzoquinone molecules maximally accept two electrons each, only 32 electrons can be drawn from the acceptor side unless reduced benzoquinones are reoxidized. The gradual increase in activity after the initial steep rise at an interval of 4 s can be explained by a slow reoxidation of the reduced benzoquinones during long dark intervals, whereas the gradual increase up to 100 flashes at a 0.01-s interval may be ascribed to an inefficient reduction of benzoquinone at too short dark intervals. The decline in activity from the maximum level with increasing flash number as seen at intervals of 0.1 and 0.5 s would be due to photoinactivation of once photoactivated PSII complexes (Miyao & Inoue, 1991a).

Quantum Requirement for Photoactivation. Since quite a high photoactivation yield could be achieved, we next examined the minimum flash number required for the photoactivation. In these experiments, 20 μM phenyl-BQ was used as an effective electron acceptor, and the flash interval of 0.5 s was employed as an optimum. To raise the flash yield as much as possible, Mn^{2+} concentration was increased to 10 mM, and Ca^{2+} was excluded from the photoactivation mixture but added after flash illumination: under these conditions the activity recovery by four flashes was higher by 5–10% than that when 2 mM Mn^{2+} and 20 mM Ca^{2+} were present during illumination (data not shown).

Figure 3 shows the dependence on flash number of restoration of oxygen-evolving capacity. Traces of output of the oxygen electrode during assay of oxygen evolution were directly presented. To enlarge tiny differences in oxygen-evolving activity, the sample concentration on the assay was increased from 10 to 30 μg/mL, though the intensity of actinic light became undersaturated. Even without flash illumination, our NH_2OH-treated PSII membranes exhibited a residual oxygen-evolving activity (trace b), which disappeared in the presence of DCMU (not shown). The slope of the trace did not change at all after illumination with a single flash (trace c). After illumination with two flashes, however, the slope was

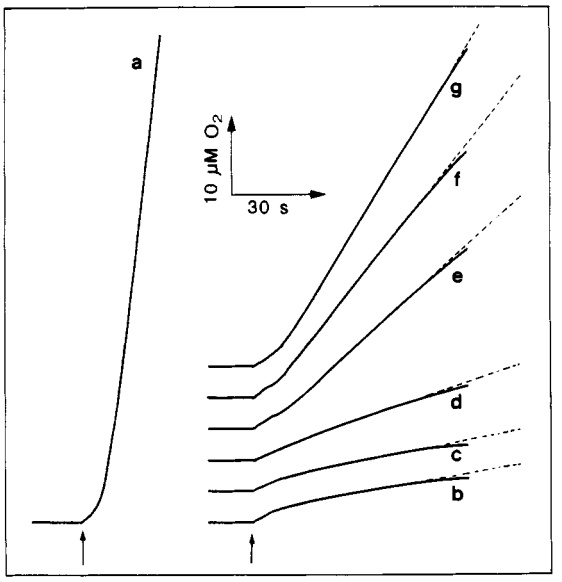

FIGURE 3: Dependence on flash number of the appearance of oxygen-evolving capacity in NH_2OH-treated PSII membranes. NaCl-treated or NH_2OH-treated PSII membranes were suspended in 10 mM Mn^{2+}, 1.2 M Cl^-, and 20 μM phenyl-BQ and kept in darkness or illuminated with flashes at 0.5-s intervals. Then the suspension was supplemented with 100 mM Ca^{2+} and kept in darkness for 15 min at 25 °C. After dilution, the oxygen-evolving activity was measured in the presence of 1.2 mM EDTA and 23-kDa and 16-kDa proteins at 30 μg of Chl/mL. Arrows indicate the time at which actinic light was turned on. Dotted straight lines represent slopes used to calculate oxygen-evolving activity. (a) NaCl-treated membranes incubated in darkness; (b) NH_2OH-treated membranes incubated in darkness; (c–g) NH_2OH-treated membranes illuminated with one to five flashes, respectively.

slightly but significantly increased (trace d). The slope became remarkably steepened upon illumination with one more flash (trace e). The activities calculated from the slope are plotted against flash number in Figure 4a. It is clearly seen that the oxygen-evolving activity was increased after illumination with two flashes and then further increased upon additional flashes.

We next examined the effects of flashes fired in the absence of Mn^{2+}. NH_2OH-treated PSII membranes were illuminated with one or two flashes in the presence of phenyl-BQ but absence of Mn^{2+} and then supplemented with Mn^{2+} and further illuminated with flashes. Also in these cases, the activity recovery was observed only after two or more flashes fired in the presence of Mn^{2+} (Figure 4b,c), indicating that illumination with at least two flashes in the presence of Mn^{2+} is essential for photoactivation. These observations directly confirm the two-quantum process model for photoactivation (Scheme I) and suggest that the minimum quantum requirement for photoactivation is two.

Although the flash preillumination in the absence of Mn^{2+} did not alter the minimum quantum requirement, it significantly improved the extent of activity recovery by the following two flash illumination in the presence of Mn^{2+} (lower panel of Figure 4). One preflash was most effective and almost doubled the recovery as compared to that without preflash.

Discussion

Effects of Benzoquinones on Photoactivation with Flashing Light. In previous studies of photoactivation, DCIP has been used as the best electron acceptor. In fact, DCIP showed the highest recovery in the photoactivation with continuous light (Tamura & Cheniae, 1987a; Miyao & Inoue, 1991a). When the photoactivation was effected by flashing light, however, DCIP was not very effective as compared with phenyl-BQ

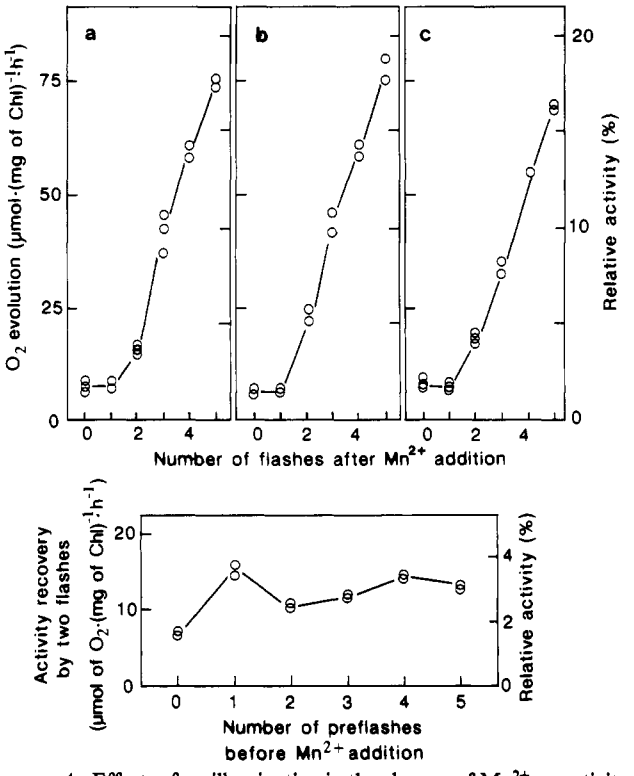

FIGURE 4: Effects of preillumination in the absence of Mn^{2+} on activity recovery by subsequent flash illumination after Mn^{2+} addition. NH_2OH-treated PSII membranes were suspended in 1.2 M Cl^- and 20 μM phenyl-BQ and kept in darkness or illuminated with designated numbers of flashes. Then the suspension was supplemented with 10 mM Mn^{2+} by adding 0.2 M $MnCl_2$ within 10 s and further illuminated with flashes. The flash interval was 0.5 s. The oxygen-evolving activity was measured after a supplement with Ca^{2+} as described in the Figure 3 legend. 100% stands for the same as that in Figure 1. Results of one to three experiments are presented. (Upper panels) Effects of preflashes on the flash-number dependence of the appearance of oxygen-evolving capacity. NH_2OH-treated membranes were kept in darkness (a) or illuminated with 1 (b) or 2 (c) flashes in the absence of Mn^{2+}, supplemented with Mn^{2+}, and further illuminated with designated numbers of flashes. (Lower panel) Plot of activity recovery after the second flash in the presence of Mn^{2+} against the number of preflashes. Activity recovery represents the difference in oxygen-evolving activity before and after flash illumination.

(Miyao & Inoue, 1991a). This led us to expect that some electron acceptors other than DCIP might dramatically raise the flash photoactivation yield.

As shown in Table I, six out of eight different benzoquinones almost doubled the activity recovery by 10 flashes at 0.25-s intervals when their concentrations were optimized. Generally, the benzoquinones that rapidly draw electrons from the acceptor side of PSII were effective in supporting the photoactivation.

The flash-interval dependences of flash photoactivation yield (Figure 1) indicate that the benzoquinones do not improve the quantum yield of the photoreactions involved in the photoactivation process but facilitate formation of the second unstable intermediate (conversion from A to C). According to Scheme I, the overall rate of its formation is determined by the relative rates of two processes, the forward rearrangement and backward decay of the first unstable intermediate (conversions of B to C and of B to A): decay of the second unstable intermediate (conversion of C to A) is much slower than the formation (see Figure 1) and does not significantly participate in the overall formation rate. If the main action of the benzoquinones were to accelerate the rearrangement, the maximum level in flash-interval dependence would reach almost the same level regardless of the presence or absence of benzoquinones, though the interval giving the maximum recovery would shift to a longer interval in the absence of benzoquinones. As seen in Figure 1, however, this is not the case. Thus, we conclude that benzoquinones suppress the decay of the first unstable intermediate and thereby raise the yield of the second unstable intermediate. Tetrabromo- and phenyl-BQs, which were most effective in photoactivation (Table I), additionally acted to suppress the decay of the second unstable intermediate (Figure 1).

Functioning Mechanism of Benzoquinones in Photoactivation. To clarify the functioning mechanism, we first examined the decay process of two unstable intermediates. It has been demonstrated that the first photoreaction involves oxidation of one Mn^{2+} to yield Mn^{3+} (Miller & Brudvig, 1990). This implies that the first unstable intermediate is a state containing one Mn^{3+} bound. The second unstable intermediate is also considered to contain one Mn^{3+} bound (Ono & Inoue, 1987; Tamura & Cheniae, 1987a). Thus, the decay of both the two unstable intermediates corresponds to rereduction of the bound Mn^{3+}. Mn^{3+} in the first intermediate is much less sensitive to exogenously added reductants than Mn^{3+} in the second one, since reductants decreased the photoactivation yield preferentially at long flash intervals, leaving the yield at short intervals unaffected (Tamura & Cheniae, 1987b; Miyao & Inoue, 1991a): in the absence of added reductants, the reduced form of artificial electron acceptors accumulated during illumination acts as reductants for Mn^{3+} (Miyao & Inoue, 1991a). As we assume the same valence of Mn^{3+} for both of the two intermediates, the different sensitivity to reductants cannot be due to difference in the oxidation state of Mn^{3+}. We speculate that the Mn^{3+} in the first intermediate resides in an intrinsic part of the PSII complex to which reductants cannot easily access, and the rearrangement to the second intermediate exposes the Mn^{3+} to the outer side of the complex. These in turn imply that redox reactions between Mn^{3+} and reductants cannot be the main cause for the decay of the first unstable intermediate. The most probable cause for the decay is a charge recombination reaction of Mn^{3+} with Q_A^- or Q_B^- as the negative counterpart. The second unstable intermediate, on the other hand, may be lost mainly by the redox reactions between Mn^{3+} and reductants. The relatively slow decay of the second intermediate (see Figure 1) might result from slow diffusion of the reductants to a close vicinity of the bound Mn^{3+}.

In NaCl-treated PSII membranes, the starting material in this study, electron transfer from Q_A to Q_B was blocked in about one-third of the PSII complexes, judging from the profile of thermoluminescence glow curve after excitation with a single flash (data not shown). In the PSII complexes in which electron transfer from Q_A to Q_B is blocked, Q_A^- stably remains and acts as a negative counterpart of charge recombination reaction with Mn^{3+}. In these complexes exogenously added benzoquinones can directly oxidize Q_A^- (Tanaka-Kitatani et al., 1990). On the other hand, in the PSII complexes in which the Q_A-to-Q_B electron transfer is intact, benzoquinones rapidly reoxidize Q_A^-, probably by binding to the Q_B binding site (Zimmermann & Rutherford, 1986; Diner & Petrouleas, 1987), and Q_B^- (the reduced benzoquinone on the Q_B binding site) is efficiently reoxidized or replaced by another benzoquinone molecule (Tanaka-Kitatani et al., 1990). Thus, it is quite likely that benzoquinones effectively minimize the charge recombination reactions by oxidizing Q_A^- and Q_B^- and thereby stabilize the first unstable intermediate.

The above considerations are compatible with our finding that the benzoquinones which rapidly draw electrons from the

acceptor side of PSII are also effective in the photoactivation. One exception was tetramethyl-BQ that exhibited a relatively high V_{max} value but no enhancement of the photoactivation (Table I). This might be due to its low affinity to the Q_B binding site: the affinity of tetramethyl-BQ is much lower than those of other benzoquinones such as dichloro- and dimethyl-BQs, judging from the benzoquinone concentrations required to mitigate the inhibition by DCMU of Q_A^- reoxidation [Kazuhiko Satoh, unpublished; also see Soll and Oettmeier (1984)]. Probably tetramethyl-BQ does not bind to the Q_B binding site and consequently cannot rapidly reoxidize Q_A^- and Q_B^-.

As for stabilization of the second unstable intermediate by tetrabromo- and phenyl-BQs, the mechanism is not clear. If the redox reaction between the bound Mn^{3+} and reductant(s) were the main cause for the decay of this intermediate, the decay rate would depend on how efficiently and easily the reducing power of reduced benzoquinones is transferred to the Mn^{3+}. Since PSII membranes contain only about three plastoquinone molecules per center (Miyao & Murata, 1987), it is unlikely that the plastoquinone mediates the redox reactions between Mn^{3+} and reduced benzoquinone. Rather, we assume that benzoquinone itself would act as the mediator. Tetrabromo- and phenyl-BQs are very hydrophobic so that they presumably cannot approach the Mn^{3+} which would be located in hydrophilic environment. We note that tetrabromo-BQ has been reported to covalently bind to thylakoid proteins (Oettmeier et al., 1987).

Quantum Requirement for Photoactivation. It has been proposed that two successive photoreactions are sufficient to reconstitute the functional Mn cluster (Radmer & Cheniae 1971, 1977; Inoue et al., 1975; Ono & Inoue, 1987). This view has been indirectly supported by experiments using repetitive illumination with flash clusters containing different numbers of flashes, which showed that the flash clusters containing more than two flashes were equally effective in the photoactivation (Ono & Inoue, 1987). In the present study, we could directly prove the model by showing that the photoactivation could be achieved by illumination with two flashes (Figures 3 and 4). We also found that preillumination improved the photoactivation yield (Figure 4). This preillumination effect would result from the competition between photooxidation of Mn^{2+} and photooxidation of Y_D as proposed by Miller and Brudvig (1990). In fact, about 50% of Y_D remains in reduced form in NH_2OH-treated PSII membranes in darkness [see Ono and Inoue (1991b)]. Preillumination will oxidize Y_D and thereby increase the quantum yield of Mn^{2+} photooxidation by the first flash.

Even under our optimized conditions, the quantum yield of photoactivation was still low. We roughly estimated the quantum yield on the following assumptions: (1) photons are utilized by the PSII complex for the photoactivation at an equal quantum yield (ϕ) at every flash illumination in the presence of Mn^{2+}, and (2) two effective photons accomplish the photoactivation. Then the activity recovery after two flashes is given by ϕ^2, and ϕ is obtained as the square root of the recovery. The quantum yield thus estimated from experimental results in Figure 4 was 0.13 without preflash and increased to 0.19 after one preflash. Recently, Ono and Inoue (1991a,b) have proposed that a histidine residue(s) at the donor side of PSII is photooxidized in Mn-depleted PSII complex and that the oxidized histidine acts to oxidize Mn^{2+} in the photoactivation process: they have shown that the oxidized histidine gives rise to the thermoluminescence A_T band and that the capability of A_T band formation correlates well with the photoactivation yield. The quantum yield of histidine photooxidation (A_T band formation) was about 0.6 at −23 °C (Ono & Inoue, 1991a). Judging from the temperature dependence of A_T band formation (Koike et al., 1986), the oxidized histidine is unstable and rapidly lost at room temperature. On the other hand, the redox reaction between Mn^{2+} and the oxidized histidine appears highly efficient, since exogenously added Mn^{2+} almost completely suppressed the A_T band (Ono & Inoue, 1991a). Thus, we speculate that the limited quantum yield of photoactivation arises from the low stability of the putative oxidized histidine residue.

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Registry No. DCIP, 956-48-9; tetrabromo-BQ, 488-48-2; phenyl-BQ, 363-03-1; 2,6-dichloro-BQ, 697-91-6; 2,5-dichloro-BQ, 615-93-0; tetrachloro-BQ, 118-75-2; 2,5-dimethyl-BQ, 137-18-8; O_2, 7782-44-7; Mn, 7439-96-5.

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Using Saturation-Recovery EPR To Measure Distances in Proteins: Applications to Photosystem II†

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ABSTRACT: The stable tyrosine radical $Y_D^\bullet$ (tyrosine 160 in the D2 polypeptide) in photosystem II (PSII) exhibits nonexponential electron spin-lattice relaxation transients at low temperature. As previously reported, the tetranuclear Mn complex in PSII significantly enhances the spin-lattice relaxation of $Y_D^\bullet$. However, in Mn-depleted PSII membranes, the spin-lattice relaxation transients of $Y_D^\bullet$ are also nonexponential, and progressive power saturation ($P_{1/2}$) experiments show that it does not behave like an isolated tyrosine radical. A model is developed to treat the interaction of two paramagnets in a rigid lattice at a fixed distance apart but with a random orientation in a magnetic field. This model describes the spin-lattice relaxation of a radical in proximity to another paramagnetic site in terms of three relaxation rate constants: the "intrinsic" relaxation rate, the relaxation rate due to scalar exchange, and the relaxation rate due to dipole-dipole interactions. The intrinsic and the scalar exchange relaxation rates are isotropic and together contribute a single rate constant to the spin-lattice relaxation transients. However, the dipolar relaxation rate is orientation dependent. Each orientation contributes a different dipolar relaxation rate constant to the *net* spin-lattice relaxation rate constant. The result is a superposition of single-exponential recoveries, each with a different net rate constant, causing the observed saturation-recovery transients to be non-(single)-exponential. Saturation-recovery relaxation transients of $Y_D^\bullet$ are compared with those of a model tyrosine radical, generated by UV photolysis of L-tyrosine in a borate glass. From this comparison, we conclude that scalar exchange does not make a significant contribution to the spin-lattice relaxation of $Y_D^\bullet$ in Mn-depleted PSII. We account for the nonexponential relaxation transients obtained from $Y_D^\bullet$ in Mn-depleted PSII membranes in terms of dipolar-induced relaxation enhancement from the non-heme Fe(II). From simulations of the spin-lattice relaxation transients, we obtain the magnitude of the magnetic dipolar interaction between $Y_D^\bullet$ and the non-heme Fe(II), which can be used to calculate the distance between them. Using data on the non-heme Fe(II) in the reaction center of *Rhodobacter sphaeroides* to model the non-heme Fe(II) in PSII, we calculate a $Y_D^\bullet$-Fe(II) distance of $\geq$38 Å in PSII. This agrees well with the distance predicted from the structure of the bacterial reaction center.

Researchers have noted in several studies of multisite redox enzymes that a paramagnetic species of interest showed nonexponential (or at least non-single-exponential) spin-lattice (T_1) relaxation kinetics at temperatures under 100 K. Examples include Cu_A in cytochrome oxidase (Scholes et al., 1984) and the tyrosine radical $Y_D^\bullet$ in PSII[1] (Britt et al., 1987; Evelo et al., 1989; Beck et al., 1990). We suggest that in both cases the source of the observed nonexponential spin-lattice relaxation kinetics is an orientationally dependent dipole-dipole interaction between the observed spin system and one or more of the endogenous paramagnetic centers in these enzymes. In this paper, we demonstrate that the unusual spin-lattice relaxation kinetics of $Y_D^\bullet$ in manganese-depleted PSII arise directly from its dipolar interaction with another paramagnetic center, probably the non-heme Fe(II).

PSII contains a remarkably stable tyrosine radical, $Y_D^\bullet$, which exhibits the well-studied EPR spectrum known as signal II_s (Babcock et al., 1989). Although it is now known that $Y_D^\bullet$ is located at tyrosine 160 in the D2 polypeptide (Debus et al., 1988; Vermaas et al., 1988), the location of Y_D with respect to the other components of the O_2-evolving center (OEC) and to the rest of the PSII reaction center is not well established.

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[1] Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; OEC, O_2-evolving center; PSII, photosystem II; $Y_D^\bullet$, tyrosine radical in photosystem II giving rise to signal II_s.

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