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An improved procedure for photoactivation of photosynthetic oxygen evolution: Effect of artificial electron acceptors on the photoactivation yield of NH_2OH-treated wheat Photosystem II membranes

Mitsue Miyao and Yorinao Inoue

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako (Japan)

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The conditions for photoactivation of oxygen evolution in Mn-depleted wheat Photosystem II (PS II) membranes reported by Tamura and Cheniae (Tamura, N. and Cheniae, G. (1987) Biochim. Biophys. Acta 890, 179–194) were examined in more detail. Treatment with 1.0 mM NH_2OH of wheat PS II membranes lacking the extrinsic 23 kDa and 16 kDa proteins removed almost all the Mn atoms from the PS II complex and reduced the oxygen-evolving activity to less than 5% of that before the treatment. About a half of the lost activity was restored by a 60 min incubation of the treated membranes with supplements of 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^-/100 μM 2,6-dichlorophenolindophenol (DCIP) under weak light illumination according to the protocol of Tamura and Cheniae. On lowering the DCIP concentration during illumination, the rate of photoactivation was accelerated and a much higher activity was restored: with 5–20 μM DCIP the activity restoration reached about 80% of the original level in 15 min. Dependence of photoactivation yield by repetitive flashes on flash interval indicated that a low concentration (5 μM) of DCIP increased the yield without affecting the rates of light-driven formation and decay of the intermediate state, while DCIP above 5 μM specifically accelerated its decay, resulting in a lower yield. Phenyl-*p*-benzoquinone at 0.4 mM also stimulated the flash photoactivation yield, but the maximum activity restoration under continuous illumination was at most 50% of the original. This limited restoration is mainly due to light-induced inactivation of photoactivated centers in the presence of the quinone. Characteristics of PS II electron acceptors required for photoactivation are discussed.

Introduction

Photosynthetic oxygen evolution is catalyzed by the Mn cluster which is bound to the PS II complex at the lumenal surface of thylakoid membranes [1,2]. The Mn cluster, consisting of four Mn atoms of valences II and III or III and IV (for a review, see Ref. 3), is dissociated from the complex by treatment with NH_2OH, Tris or alkaline pH with concomitant inactivation of oxygen evolution [4], and can be reassembled in the inactivated complex by incubation with Mn^{2+} under illumination to restore oxygen evolution [5]. This light-dependent reconstitution of the Mn-cluster, so-called photoactivation of oxygen evolution, is driven by at least two successive photoreactions in PS II [6–8] and generally considered to involve oxidation of Mn^{2+} by PS II followed by assembly of the oxidized Mn into a functionally active Mn cluster in the water-oxidation site of the PS II complex [7,9–12]. This process requires not only Mn^{2+} but also Ca^{2+} [13–16] and Cl^- [17] as essential inorganic cofactors.

The photoactivation of oxygen evolution has been studied using a wide variety of materials: angiosperm leaves grown under intermittent illumination [8,18], dark-grown gymnosperm leaves [19], dark-grown algae [7], intact chloroplasts from leaves grown under intermittent illumination [9,10,15], thylakoid membranes treated with Tris [13,14], and PS-II-enriched membrane preparation treated with NH_2OH [11]. Among these, PS

Abbreviations: Chl, chlorophyll; DCIP and $DCIPH_2$, 2,6-dichlorophenolindophenol in oxidized and reduced form, respectively; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; PBQ, phenyl-*p*-benzoquinone; PS II, Photosystem II.

Correspondence: M. Miyao, Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan.

II membranes are the most useful to study, since the conditions of ions and cofactors required for the process can be easily controlled exogenously.

Tamura and Cheniae [11] first succeeded in photoactivating oxygen evolution in NH_2OH-treated PS II membranes by illuminating the treated membranes in the presence of 1 mM $MnCl_2$, 50 mM $CaCl_2$ and 100 μM DCIP. In their system, however, recovery of oxygen evolution was not always high. In this study, we reexamined in more detail the conditions for photoactivation using wheat PS II membranes, and found that the yield of photoactivation can reach as high as 80% of the original by optimizing the DCIP concentration.

Materials and Methods

Preparation of PS II membranes and extrinsic proteins

Thylakoid membranes were prepared from 8-day-old wheat seedlings grown under continuous light from fluorescent lamps [9]. PS II membranes were prepared with Triton X-100 from the thylakoids as previously reported [9] with slight modifications as follows. The thylakoids were suspended in 10 mM NaCl/5 mM $MgCl_2$/0.4 M sucrose/25 mM Mes-NaOH (pH 6.5) at 1.0 mg Chl $\cdot$ ml^{-1}. An aqueous solution of Triton X-100 (20%, w/v) was added to the suspension to give a Triton-to-Chl ratio of 20 : 1 (w/w). After incubation for 3 min at 0°C, the suspension was centrifuged at 2000 $\times$ g for 3 min, and then the supernatant at 47 000 $\times$ g for 30 min. The pellet from the supernatant was suspended in the same medium, centrifuged at 500 $\times$ g for 2 min, and then at 40 000 $\times$ g for 20 min, and the resultant pellet of PS II membranes were suspended in 10 mM NaCl/0.4 M sucrose/25 mM Mes-NaOH (pH 6.5) (hereafter designated medium A) containing 30% (v/v) ethylene glycol and stored in liquid nitrogen.

Before use, the PS II membranes were thawed and washed three times with medium A by centrifugation at 40 000 $\times$ g for 10 min and resuspension. The extrinsic 23-kDa and 16-kDa proteins were removed from the PS II membranes by treatment with 1.5 M NaCl in darkness [20]. The NaCl-treated membranes were collected by centrifugation at 40 000 $\times$ g for 10 min, resuspended after one wash in medium A, and stored in liquid nitrogen. Before being treated with NH_2OH, the NaCl-treated membranes were thawed and kept in darkness for 1 h. Then, the membranes were suspended in medium A containing 1.0 mM NH_2OH at 0.5 mg Chl $\cdot$ ml^{-1} and incubated for 5 min in darkness. The NH_2OH-treated membranes were collected by centrifugation at 40 000 $\times$ g for 10 min and suspended in medium A containing 2.0 mM EDTA at about 0.1 mg Chl $\cdot$ ml^{-1}. The membranes were again collected by centrifugation, washed once with medium A as above and finally suspended in medium A. For preparation of extrinsic proteins, the 23 kDa and 16 kDa proteins were extracted with 1.5 M NaCl from untreated PS II membranes, concentrated by ultrafiltration with an Amicon YM 10 Diaflo membrane, and dialyzed against 10 mM Mes-NaOH (pH 6.5). All the procedures were done at 0–4°C.

Photoactivation

Photoactivation of oxygen evolution of NH_2OH-treated PS II membranes was done according essentially to Tamura and Cheniae [11]. The NH_2OH-treated membranes suspended in 0.1 or 1.0 mM $MnCl_2$/20 mM $CaCl_2$/110 mM NaCl/0.4 M sucrose/25 mM Mes-NaOH (pH 6.5) containing designated additions at 0.25 mg Chl $\cdot$ ml^{-1} were put in a flat glass tray of 14 mm in diameter. For photoactivation by continuous light, the suspension less than 1 ml was gently shaken under illumination with fluorescent lamps at an almost saturating light intensity of 33 $\mu E \cdot m^{-2} \cdot s^{-1}$ at 25°C. For photoactivation by flashing light, the suspension (0.4 ml) was illuminated with 4-μs xenon flashes at saturating intensity while gently stirring at 25°C. Light saturation of the flash was confirmed by use of a neutral-density filter (75%). After illumination the suspension was diluted with medium A containing the 23 kDa and 16 kDa proteins sufficient to maximize oxygen-evolving activity. Then, the activity was measured at 25°C with 0.45 mM PBQ as an electron acceptor using a Clark-type oxygen electrode. Final Chl concentrations of assay mixtures were 5 and 10 $\mu g \cdot ml^{-1}$ when photoactivated by continuous and flashing light, respectively. Activity recovery was expressed by the oxygen-evolving activity of photoactivated membranes as percentage of the activity of NaCl-treated PS II membranes measured in medium A containing 10 mM $CaCl_2$. The activities of NaCl-treated membranes measured in the presence of 10 mM $CaCl_2$ were equivalent to those of untreated PS II membranes.

Measurement of DCIP concentration

The concentration of the oxidized form of DCIP remaining after illumination of a suspension of NaCl-treated or NH_2OH-treated PS II membranes was determined as follows. A PS II membrane suspension containing 0.25 mg Chl $\cdot$ ml^{-1} and DCIP was diluted 12.5-fold with medium A which had been degassed with nitrogen, and then, 1/250 vol. of 20 mM sodium ascorbate/10 mM Mes-NaOH (pH 6.5) was added to the suspension. The concentration of oxidized form of DCIP was calculated from the absorbance difference between before and after the ascorbate addition based on the extinction coefficient of DCIP at 600 nm of 18.1 $mM^{-1} \cdot cm^{-1}$ at pH 6.5 (Ref. 21). Fluctuations of the dilution factor were corrected by use of Chl absorbance at the red absorption peak measured after complete reduction of DCIP. Handling of the suspension was done under dim green light.

Other methods

DCIP (sodium salt) was freshly dissolved in 10 mM Mes-NaOH (pH 6.5) at about 5 mM. The DCIP solution was kept at 25°C until use, since a slight cooling resulted in appearance of insoluble educts which led to a decrease in absolute DCIP concentration. Accurate DCIP concentration was determined from the absorbance at 600 nm using the extinction coefficient described above. PBQ used for photoactivation was purified by rinsing the powder-like crystals with ethanol for a few seconds, followed by drying under vacuum. Chl was determined according to Arnon [22]. Low-temperature EPR spectra were recorded at 6 K as described previously [23]. Mn content was determined by atomic absorption at 279.5 nm with a flameless atomic absorption spectrometer (Shimadzu, AA-640-13 and GFA-3). Samples prepared in 0.5% HNO_3 were dried at 160°C for 40 s, ashed at 900°C for 30 s and atomized at 2500°C for 6 s.

Results

Previous studies showed that Mn^{2+} is incorporated into the PS II complex when NH_2OH-treated PS II membranes are incubated only with $MnCl_2$ under illumination and that Ca^{2+} is necessary for the incorporated Mn to express oxygen-evolving activity [11,16,24]. In the present experiments 20 mM Ca^{2+} was included in all the incubation mixture, because our preliminary investigations revealed that 20 mM Ca^{2+} could maximize photoactivation at every concentration of Mn^{2+} and DCIP tested (data not shown).

NH_2OH treatment of NaCl-treated PS II membranes reduced the oxygen-evolving activity to less than 5% of that before the treatment. When the NH_2OH-treated PS II membranes were incubated under illumination with 1.0 mM Mn^{2+} but without DCIP, the oxygen-evolving activity recovered to 35% of the original level within 15 min and remained constant (Fig. 1A). Addition of 100 μM DCIP to incubation mixture, according to the previously reported protocol [11], enhanced the activity recovery: after initial rise to 40% within 15 min the activity gradually increased to reach 60% in 60 min. However, much more enhanced activity recovery could be attained when DCIP concentration was reduced. On lowering DCIP concentration, the initial rise in activity became more rapid and the maximum level higher (Fig. 1A). With 6 μM DCIP about 80% of the original activity was recovered after a 15-min illumination. The extent of activity recovery gradually decreased with prolonged illumination, and its course roughly coincided with that of activity decrease found when NaCl-

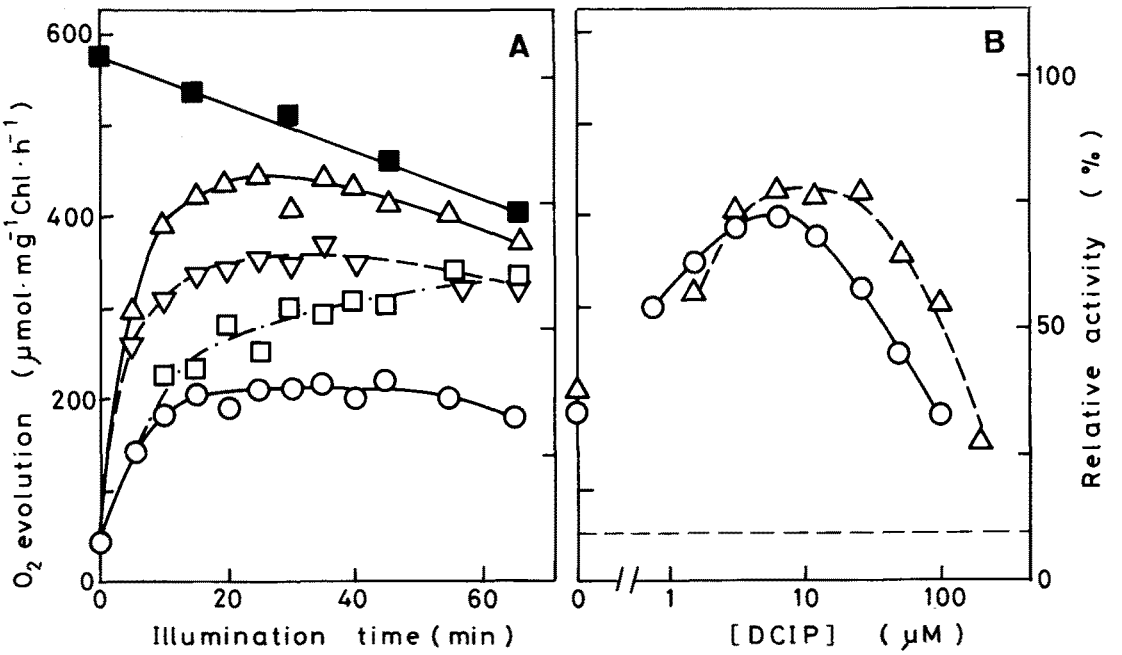

Fig. 1. Effects of DCIP concentration on photoactivation of oxygen evolution by continuous illumination. (A) Time-course of restoration of oxygen-evolving activity during photoactivation treatment with various DCIP concentrations. NH_2OH-treated (open symbols) or NaCl-treated PS II membranes (closed symbols) were incubated with 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^- containing designated concentrations of DCIP under continuous illumination. After a designated time the suspension was diluted with medium A containing the extrinsic 23 kDa and 16 kDa proteins and oxygen-evolving activity was measured. ○, no; △, 6 μM; ▽, 50 μM; □, ■, 100 μM DCIP. (B) Dependence on DCIP concentration of the maximum activity restoration. NH_2OH-treated PS II membranes were incubated with 0.1 (○) or 1.0 mM (△) Mn^{2+}, Ca^{2+}, Cl^- and DCIP under continuous illumination as above and oxygen-evolving activity was measured every 5 min. Maximum activities attained during the incubation for 60 min were plotted. The horizontal broken line represents the activity of NH_2OH-treated PS II membranes before illumination. 100% corresponds to the activity of NaCl-treated PS II membranes measured in medium A containing 10 mM $CaCl_2$.

treated PS II membranes were incubated with Mn^{2+}, Ca^{2+}, Cl^- and DCIP under illumination (Fig. 1A). Thus, we conclude that oxygen evolution by NH_2OH-treated PS II membranes can be almost fully reactivated by the photoactivation treatment with 6 μM DCIP.

Fig. 1B shows the titration of DCIP concentration for two different Mn^{2+} concentrations employed for photoactivation by continuous light. In the presence of 1.0 mM Mn^{2+}, the activity recovery showed maximum at 5–20 μM DCIP. When Mn^{2+} concentration was reduced to 0.1 mM, the optimum DCIP concentration was found at a lower concentration. At both Mn^{2+} concentrations the maximum activity recovery was close to 80% of the original level.

Changes in Mn content and oxygen-evolving activity on NH_2OH treatment and subsequent photoactivation treatment with various DCIP concentrations are shown in Fig. 2A. Previous reports showed that NH_2OH treatment removed three-fourths of Mn from PS II complex, leaving about one Mn per PS II remaining bound [25,26]. In contrast, the NH_2OH-treated PS II membranes used in this study were almost completely depleted of Mn. This difference may have resulted from the relatively harsh wash of the membranes after NH_2OH treatment in our protocol (see Materials and Methods). Similar harsh washes were also applied to photoactivated membranes before Mn determination. These washes caused partial loss of oxygen-evolving activity by 10–20%, but the dependence of activity recovery on DCIP concentration was well retained (Fig. 2A). This dependence curve almost completely coincided with that for Mn incorporation, indicating that Mn rebinds to depleted membranes in proportion to activity recovery. We can thus conclude that the oxygen-evolving activity restored by this photoactivation treatment properly reflects the reconstitution of active Mn cluster in the PS II complex. Plot of activity recovery against Mn incorporation gave a straight line (Fig. 2B). Extrapolation of this line to 3.5 Mn/220 Chl, the Mn abundance in original PS II membranes, suggests that about 85% of the original activity can be recovered if all of the lost Mn atoms are rebound to depleted membranes. The multiline EPR signal characteristic of the S_2 state was also regenerated by this photoactivation treatment (Fig. 3).

The effects of DCIP on photoactivation yield by repetitive flashes at different intervals were investigated (Fig. 4A). At every concentration of DCIP the dependence of photoactivation yield on flash interval exhibited a bell-shaped curve as previously reported [6–8]. According to the previously proposed models involving a two-quantum process [6,8,10], rise and decay portions of the curve were interpreted as corresponding to light-driven formation of the putative intermediate state assumed between two photoreactions, and its spontaneous decay in darkness, respectively. In the absence of DCIP

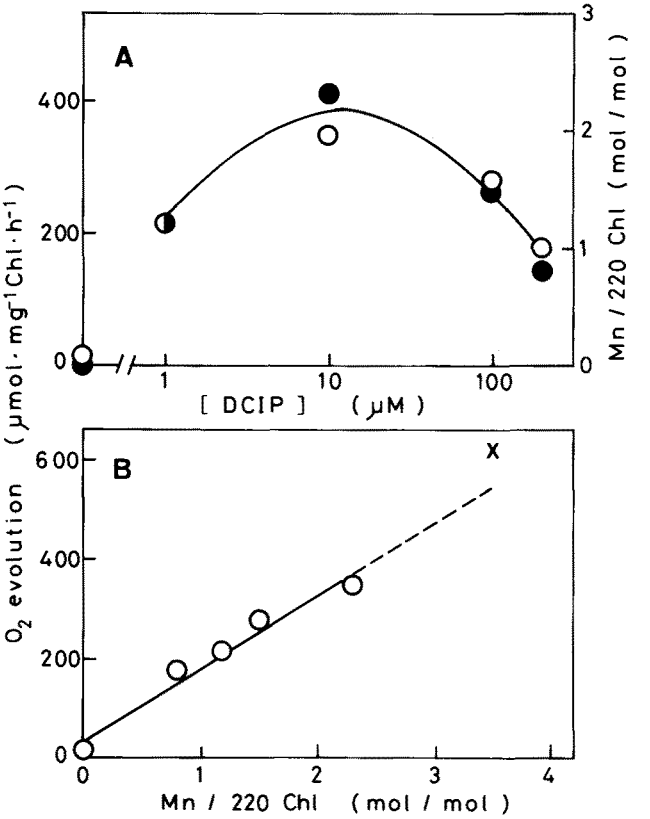

Fig. 2. Rebinding of Mn to NH_2OH-treated PS II membranes by photoactivation treatment. (A) Changes in Mn content and oxygen-evolving activity of NH_2OH-treated membranes after photoactivation with various DCIP concentrations. NH_2OH-treated membranes were incubated with 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^- containing designated concentrations of DCIP under continuous illumination for 20 min. Then, the membranes were collected by centrifugation, washed once with medium A containing 2.0 mM EDTA and twice with medium A. The oxygen-evolving activity was measured in medium A with supplements of 10 mM $CaCl_2$ and the extrinsic 23 kDa and 16 kDa proteins. Closed symbols, Mn content; open symbols, oxygen-evolving activity. Symbols on the ordinate represent values for the NH_2OH-treated membranes before the illumination. (B) Correlation between Mn content and oxygen-evolving activity. Data in (A) were replotted. A cross represents the NaCl-treated PS II membranes whose Mn content and oxygen-evolving activity were 3.5/220 Chl (mol/mol) and 620 μmol/mg Chl per h, respectively. Data of the NH_2OH-treated PS II membranes collected before and after illumination were approximated to a straight line by the least squares methods.

the yield of photoactivation showed a low maximum at an interval of 4 s. When 5 μM DCIP was added, the yield was significantly raised at all intervals longer than 0.01 s accompanied by a slight short-shift in the interval giving the maximum yield. However, the bell-shaped response on flash interval was not much affected. This implies that DCIP raises the photoactivation yield with no significant influence on the rates of formation and decay of the intermediate state. With DCIP concentrations higher than 5 μM, however, the yield at intervals longer than 0.5 s was selectively suppressed and consequently the interval for maximum yield was signifi-

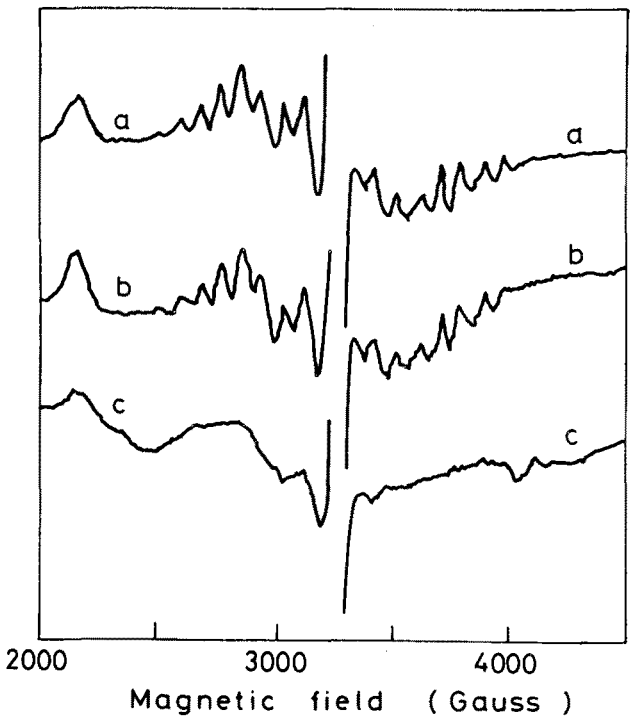

Fig. 3. Restoration of the S_2-multiline EPR signal by photoactivation treatment. NH_2OH-treated PS II membranes were incubated with 0.1 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^-/5 μM DCIP under continuous illumination or in darkness for 30 min and collected by centrifugation. PS II membranes were finally suspended in medium A containing 5 mM $CaCl_2$/2 mM EDTA/0.4 mM PBQ. The suspension was illuminated at 210 K for 4 min and EPR spectrum was recorded at 6 K. (a) NaCl-treated membranes, no incubation; (b) NH_2OH-treated membranes incubated in the light; (c) NH_2OH-treated membranes incubated in darkness. Chl concentrations were 3.1, 2.9, and 2.8 mg·ml^{-1} and oxygen-evolving activities measured as in Fig. 2 were 610, 400 and 20 μmol/mg Chl per h for (a), (b) and (c), respectively. Instrument settings: microwave power, 0.4 mW; microwave frequency, 8.95 GHz; modulation frequency and amplitude, 100 kHz and 20 G, respectively.

cantly shortened. This suggests that DCIP at higher concentrations does not affect the formation of the intermediate but accelerates its decay in darkness.

To examine whether the oxidized or reduced form of DCIP destabilizes the intermediate, effects of simultaneous addition of ascorbate and DCIP on the flash-interval dependence were investigated (Fig. 4B). The addition of 0.6 μM ascorbate to photoactivation mixture containing 15 μM DCIP, that is equivalent to reduction of 0.6 μM DCIP prior to illumination, decreased the photoactivation yield only at intervals longer than 0.5 s in a way similar to that observed when DCIP concentration was increased. With 1.4 μM ascorbate the yield at the interval of 0.5 s was also decreased. Thus, we conclude that the reduced but not oxidized form of DCIP stimulates the decay of the intermediate state. This is consistent with previous works [10,27] that reducing reagents such as NH_2NH_2, hydroquinone and ascorbate destabilize the intermediate.

If the reduced form of DCIP ($DCIPH_2$) but not its oxidized form acts to destabilize the intermediate, the accelerated decay of the intermediate at higher DCIP concentrations (Fig. 4A) can be interpreted as due to accumulation of $DCIPH_2$. Accordingly, we tried to directly measure the amount of $DCIPH_2$ accumulated in the photoactivation mixture (Fig. 5). When NH_2OH-treated PS II membranes were illuminated with 100 flashes at 1-s intervals, $DCIPH_2$ accumulation was as high as a few micromolar, irrespective of DCIP concentration up to 100 μM (Fig. 5A). When, however, NaCl-treated PS II membranes capable of oxygen evolution were similarly illuminated, a substantial accumulation of $DCIPH_2$ was detected: the amount of accumulated $DCIPH_2$ increased with DCIP concentration up to 25 μM and then decreased at higher concentrations. This decrease above 25 μM DCIP may be ascribed to the ADRY (acceleration of the deactivation reactions

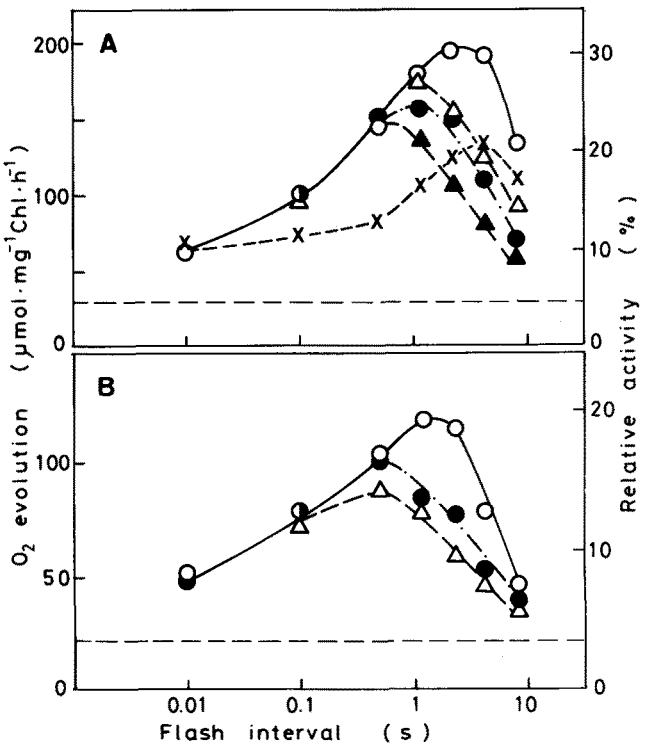

Fig. 4. Effects of DCIP on photoactivation by repetitive flashes at various intervals. NH_2OH-treated PS II membranes were suspended in 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^- containing designated additions and incubated in darkness for 1–2 min. Then, the suspension was illuminated with 100 flashes at designated intervals and oxygen-evolving activity was measured after dilution. Averages of three or four measurements are presented. The horizontal broken line and 100% stand for the same as those in Fig. 1. (A) Effects of DCIP concentration. NH_2OH-treated membranes were illuminated in the presence of no (×), 5 μM (○), 10 μM (△), 25 μM (●), or 50 μM (▲) DCIP. (B) Effects of reduced form of DCIP. NH_2OH-treated membranes were illuminated in the presence of 15 μM DCIP and designated concentrations of sodium ascorbate. Sodium ascorbate was taken from a stock solution containing 10 mM Mes-NaOH (pH 6.5). Under these conditions ascorbate stoichiometrically reduced DCIP in darkness. ○, no; ●, 0.6 μM; △, 1.4 μM sodium ascorbate.

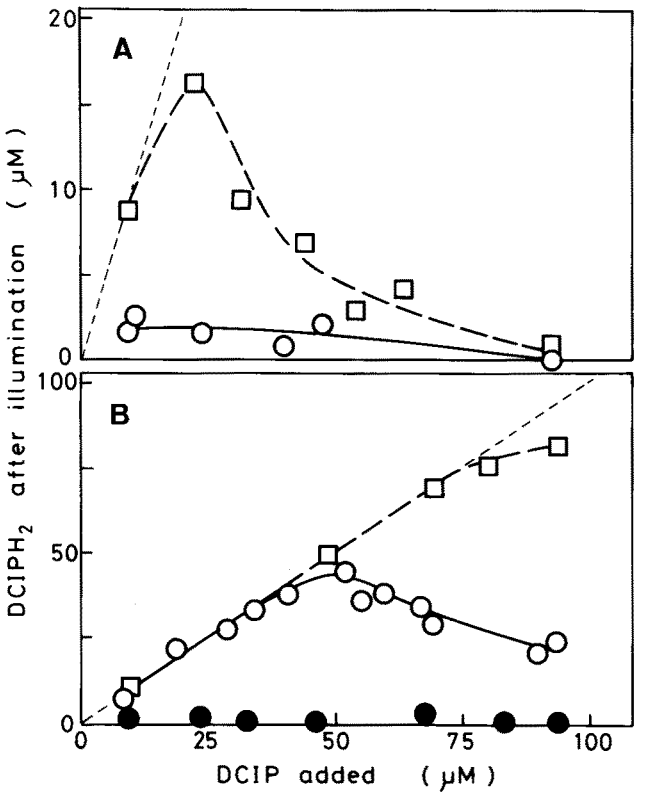

Fig. 5. Reduction of DCIP during photoactivation treatment of PS II membranes. NH_2OH-treated (circles) or NaCl-treated (squares) PS II membranes were suspended in 1.0 mM Mn^{2+}/150 mM Cl^- containing designated concentrations of Ca^{2+} and DCIP, and illuminated with 100 flashes at 1-s intervals or with continuous light for 5 min. Concentration of oxidized form of DCIP in the suspension was determined from the absorbance change at 600 nm induced by addition of excess ascorbate (see Materials and Methods), and difference between DCIP concentrations before and after illumination was taken as $DCIPH_2$ concentration formed by illumination. Measurement error was about ± 2 μM. (A) $DCIPH_2$ concentration after flash illumination. (B) $DCIPH_2$ concentration after continuous illumination. Open symbols, 20 mM Ca^{2+}; closed symbols, no Ca^{2+} was present during illumination.

of the water-splitting enzyme system Y) effect of DCIP [28], which probably involves re-reduction of higher S states by $DCIPH_2$. It is not likely that $DCIPH_2$ formed by illumination was lost by autoxidation during the assay protocol used here, since the $DCIPH_2$ formed by continuous illumination of NaCl-treated PS II membranes well maintained its reduced form after the same protocol (Fig. 5B). When continuous illumination was used for photoactivation instead of flashing light, the amount of $DCIPH_2$ found after 5 min photoactivation increased with DCIP concentration up to 50 μM to reach a maximum and then decreased at higher DCIP concentrations. In this case, however, $DCIPH_2$ accumulation does not appear to result from photoreactions involved in reactivation of the oxygen evolution. It is more likely that $DCIPH_2$ present after the illumination was formed through the Hill reaction in the photoactivated PS II complexes. This view is supported by the observation in Fig. 1A that oxygen evolution could be significantly reactivated even in early stages (5 min) of photoactivation treatment. In agreement with this, when Ca^{2+} was omitted from the photoactivation mixture to suppress oxygen evolution by reconstituted Mn clusters [11,24], $DCIPH_2$ accumulation was not detected (Fig. 5B).

Due to technical difficulties in direct determination of $DCIPH_2$ during illumination for photoactivation, the possibilities of reoxidation of $DCIPH_2$ by some oxidized species formed on illumination of NH_2OH-treated PS II membranes could not be totally excluded. However, in experiments in Fig. 5A reoxidation of $DCIPH_2$ by the intermediate for Mn cluster might be neglected, since the decay of the intermediate state was only slight at the flash interval of 1 s (Fig. 4A). The low level of $DCIPH_2$ accumulation in photoactivation mixture can be taken as consistent with the observation that the apparent inhibitory effects of DCIP at higher concentrations on flash-interval dependence was well mimicked by the presence of about 1 μM $DCIPH_2$ (Fig. 4).

Rates of DCIP photoreduction by PS II depends on DCIP concentration in assay mixture. Since binding of DCIP to its action site limits the activity at its lower concentrations, a saturating concentration which maximizes the activity is usually employed, e.g., 50 μM DCIP at 1 μg Chl · ml^{-1}. In contrast, the DCIP concentration employed for photoactivation is much lower on Chl basis, at most 100 μM DCIP at 0.25 mg Chl · ml^{-1}, so that the rate of DCIP photoreduction might be significantly suppressed. Probably due to the low concentration of DCIP, the amount of $DCIPH_2$ accumulated by photoactivation was too limited to be detected by our method, but the amount was proportional within a few micromolar range to the initial concentration of DCIP. If we take into account that the concentration of PS II reaction center in the photoactivation mixture was about 1.3 μM, assuming a molar ratio of 220 Chl per one reaction center [29], it is not surprising that such a low concentration of $DCIPH_2$ can effectively accelerate the decay of the intermediate and consequently lower the yield of photoactivation.

Photoactivation also occurred when DCIP was replaced by other artificial PS II electron acceptors. However, the activity restoration was much less as compared with DCIP as previously shown [11]. As to phenyl-*p*-benzoquinone (PBQ) and 2,5-dichloro-1,4-benzoquinone, high concentrations, above 0.1 mM, were necessary for significant enhancement of activity recovery (Fig. 6), probably due to low solubility of these quinones in aqueous suspension. Even at their optimum concentrations, 0.4 mM, the maximum recovery was about 50% of the original. Potassium ferricyanide at 0.5 to 2 mM, on the other hand, did not enhance photoactivation at all.

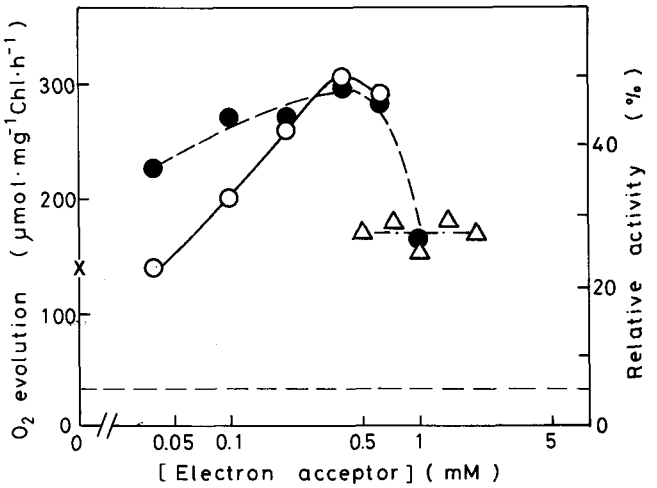

Fig. 6. Dependence on concentration of artificial electron acceptors of the maximum activity attained by photoactivation by continuous illumination. NH_2OH-treated PS II membranes were incubated with 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^- containing designated concentrations of an electron acceptor under continuous illumination and oxygen-evolving activity was measured every 5 min. Maximum activities attained during incubation were plotted. A cross on the ordinate represents the maximum activity attained by illumination without artificial electron acceptors. The horizontal broken line and 100% stand for the same as those in Fig. 1. ○, phenyl-*p*-benzoquinone; ●, 2,5-dichloro-1,4-benzoquinone; △, potassium ferricyanide.

Despite these limitations in activity restoration by photoactivation with continuous illumination, the properties of these artificial quinone acceptors as characterized by photoactivation under flashing light were very similar to those of DCIP. As Fig. 7 shows, PBQ at 0.4 mM increased the flash photoactivation yield at

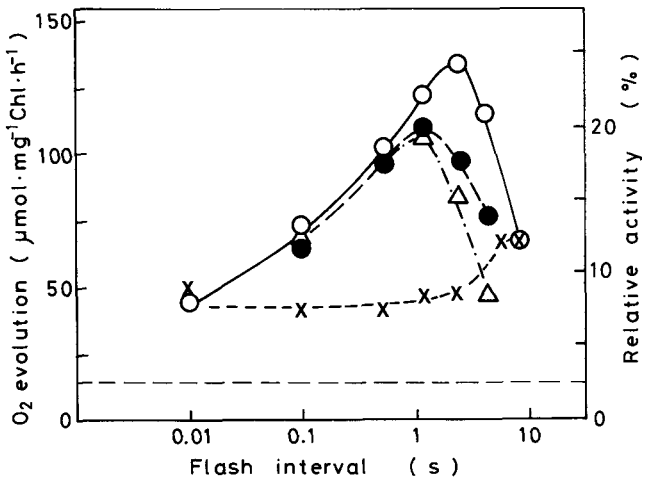

Fig. 7. Effects of phenyl-*p*-benzoquinone (PBQ) on photoactivation by repetitive flashes at various intervals. NH_2OH-treated PS II membranes were suspended in 1.0 mM Mn^{2+}/20 mM Ca^{2+}/150 mM Cl^- containing designated additions and incubated in darkness for 1–2 min. The suspension was then illuminated with 100 flashes at designated intervals and oxygen-evolving activity was measured. Averages of three or four measurements are presented. The horizontal broken line and 100% are as in Fig. 1. ×, No addition; ○. 0.4 mM PBQ; ●, 0.4 mM PBQ and 0.2 mM sodium ascorbate; △, 0.4 mM PBQ and 0.3 mM sodium ascorbate.

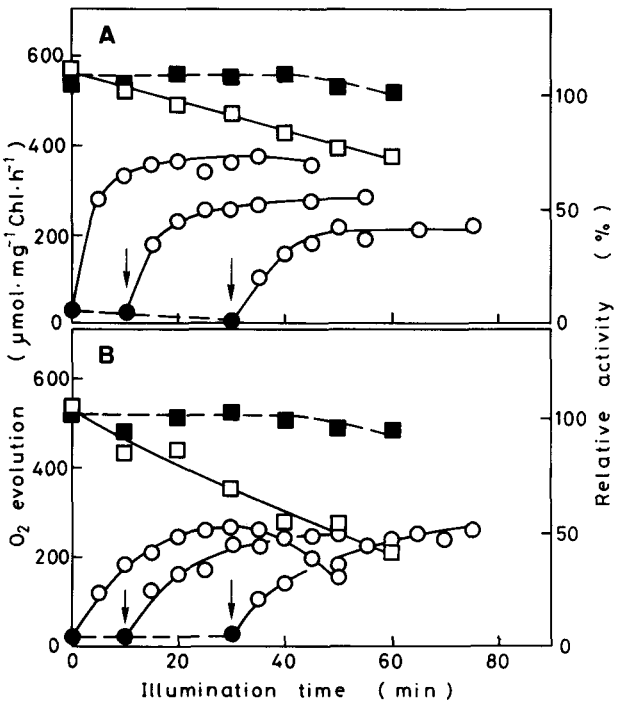

Fig. 8. Effects of illumination of PS II membranes on oxygen-evolving activity and activity restoration by subsequent photoactivation treatment. NH_2OH-treated (circles) or NaCl-treated PS II membranes (squares) were suspended in 20 mM Ca^{2+}/150 mM Cl containing 10 μM DCIP or 0.4 mM PBQ at 0.25 mg Chl·ml^{-1} and illuminated with continuous light for 0, 10 or 30 min. Then, the suspension was supplemented with 1.0 mM Mn^{2+} by adding 1/50 vol. of 50 mM $MnCl_2$ and further illuminated. Oxygen-evolving activity was measured after dilution. (A) Effects of illumination in the presence of 10 μM DCIP. (B) Effects of illumination in the presence of 0.4 mM PBQ. Open symbols, illumination in the presence of 1.0 mM Mn^{2+}; closed symbols, illumination in the absence of Mn^{2+}. Arrows indicate the time at which Mn^{2+} was added to the suspension. 100% are as Fig. 1.

every flash interval longer than 0.01 s with a slight short-shift of the interval for maximum yield, and the resulting flash-interval dependence was quite similar to that obtained with 5 μM DCIP (see Fig. 4A). When PBQ was reduced by ascorbate prior to illumination, the photoactivation yield was suppressed only at longer intervals, as has been observed for DCIP (see Fig. 4B).

These observations lead us to assume that the low recovery of activity with PBQ might be due to harmful effects of PBQ on photoactivated PS II. This assumption was examined by the experiments in Fig. 8. NH_2OH-treated PS II membranes were pre-illuminated in the absence of Mn^{2+} but in the presence of 10 μM DCIP, and after various time lapses Mn^{2+} was added. As shown in Fig. 8A, photoactivation initiated on addition of Mn^{2+} and activity reached a stationary maximum level in 10 to 20 min. Notably, however, preillumination in the absence of Mn^{2+} retarded the initial rise of activity and reduced the maximum level. After a 30-min preillumination the maximum recovery was about a half of that without preillumination. This sup-

pression of maximum recovery was not due to consumption of DCIP during the preillumination: further addition of 10 μM DCIP after preillumination failed to increase the maximum recovery, although it slightly stimulated the initial rise (data not shown). This suggests that some of the Mn-depleted PS II complexes lose the capability of photoactivation during the preillumination. Similar suppression of maximum recovery was also observed even in the presence of Mn^{2+}, if reactivation of oxygen evolution was interrupted by depletion of Ca^{2+} or Cl^- (data not shown). Klimov et al. [30] observed a similar inhibitory effect on photoactivation of preillumination in the absence of Mn^{2+} and ascribed it to inhibition of electron transport at the oxidizing side of PS II. In contrast, when 0.4 mM PBQ was employed as electron acceptor, preillumination in the absence of Mn^{2+} did not much affect the maximum recovery (Fig. 8B), suggesting that PBQ protects the Mn-depleted complexes from this light-dependent damage.

These two acceptors exhibited opposite effects on the stability of oxygen-evolving PS II membranes. When NaCl-treated PS II membranes were illuminated without Mn^{2+}, the oxygen-evolving activity remained constant for 60 min, irrespective of the type of acceptors present (Fig. 8). When Mn^{2+} was present, on the other hand, the activity gradually decreased with illumination time. With 10 μM DCIP, about 25% of the activity was lost after a 60 min illumination, whereas with 0.4 mM PBQ the activity loss was more rapid and about 60% was lost in 60 min. Our preliminary results suggest that these losses in oxygen evolution are accompanied by inhibition of electron transport from diphenylcarbazide to DCIP (data not shown). It is likely that the photoactivated PS II complexes are inevitably exposed to this type of inactivation during the photoactivation treatment. Thus, we conclude that low activity restoration with PBQ results from rapid inactivation of photoactivated complex.

Discussion

The present study revealed that the capacity of oxygen evolution of PS II membranes once completely lost by NH_2OH treatment can be almost fully restored by illuminating the treated PS II membranes in the presence of optimum concentrations of Mn^{2+}, Ca^{2+}, Cl^- and DCIP (Fig. 1). The failure in full recovery of activity may be due to damage caused by NH_2OH treatment and/or light-dependent damage (see below). The reactivation of oxygen evolution was accompanied by rebinding of Mn to depleted membranes, showing a linear correlation between the restored activity and the abundance of membrane-bound Mn (Fig. 2) as has been shown previously in the photoactivation in situ [7,31].

The maximum recovery did not much depend on the concentration of exogenous Mn^{2+} in the medium and showed almost the same level at every Mn^{2+} concentration from 0.1 to 3 mM, although a slightly longer illumination time was needed for maximum restoration at lower Mn^{2+} concentrations (data not shown). In sharp contrast, the maximum activity was markedly dependent on DCIP concentration during illumination (Fig. 1). 5–20 μM DCIP was optimum for photoactivation with 1.0 mM Mn^{2+}.

Photoactivation could proceed in PS II membranes, even though no exogenous electron acceptor was added, as has been shown previously [11]. However, the yield of photoactivation was generally low, though it varied depending on preparation. The low yield was markedly enhanced by the addition of 5 μM DCIP. Importantly, this enhancement did not much involve changes in the rates of light-driven formation of the intermediate state and its subsequent decay in darkness (Fig. 4A). It is thus inferred that only a small number of complexes among the PS II population are prepared to be photoactivated unless supplemented with an exogenous acceptor, and that DCIP enhances the yield by increasing the number of candidate complexes prepared for photoactivation. As is well known, the acceptor pool size of PS II membranes is limited to less than several electron equivalents per center [1]. This supports the above view that DCIP enhances the yield of photoactivation by substituting for the endogenous acceptor which is deficient in isolated PS II membranes. On increasing DCIP concentration above 5 μM, however, the dark decay of the intermediate becomes appreciably accelerated.

DCIP has been known to be most suitable for photoactivation [11]. When we compare Fig. 7 data with Fig. 4A, it is inferred that 0.4 mM PBQ also enhances the flash photoactivation yield as effectively as 5 μM DCIP does. Note that the flash photoactivation yield can be assumed to represent the initial rate of photoactivation. Despite, the maximum recovery with PBQ as acceptor under continuous illumination was much lower than that with DCIP (Fig. 6). Thus, some unknown factors other than the photoactivation rate are suggested to be involved in the determination of recovery level. We found that under continuous illumination photoactivation is accompanied by two inhibitory processes, and mutual interactions of these three processes determine the final recovery of oxygen evolution. One of the two inhibitory processes is impairment of Mn-depleted PS II complexes leading to an irreversible damage of PS II electron transport as proposed by Klimov et al. [30], that is to say a photoinhibition more sensitized by the absence of active Mn cluster [32]. The other process is an inactivation of oxygen evolution which occurs in PS II complexes containing active Mn cluster. The former damages the complexes before being photoactivated, whereas the latter damages the photoactivated ones.

Regarding the photoactivation with DCIP as electron acceptor, inactivation of active PS II complexes proceeds relatively slowly (Fig. 8A), so that the recovery of oxygen evolution simply depends on the relative rate of photoactivation versus impairment of Mn-depleted PS II complexes. Under these conditions, if photoactivation is much faster than the impairment, the activity recovery will reach nearly the original level. An example of this case will be the photoactivation treatment with 5–20 μM DCIP, where the half-times of both processes are roughly estimated to be a few minutes for photoactivation and about 30 min for the impairment (Fig. 8A). If photoactivation proceeds more slowly as with DCIP above 20 μM, the impairment competes with photoactivation for Mn-depleted complexes and the number of candidate complexes to be photoactivated will decrease with illumination time. In addition, a low rate of photoactivation is apt to result in further decrease in its rate due to $DCIPH_2$ which will be accumulated by the action of photoactivated complexes. Consequently, the recovery of activity will be much more suppressed.

As for photoactivation with PBQ as electron acceptor, the situations are different. In the presence of 0.4 mM PBQ, the rate of photoactivation estimated from the flash yield was comparable to that with 5 μM DCIP (Fig. 7) and the impairment process was well suppressed (Fig. 8B). However, the presence of PBQ significantly enhanced photoinactivation of active complexes (Fig. 8B), leading to re-inactivation of once photoactivated complexes. Due to this effect, the activity recovery with PBQ as acceptor is slow in kinetics and low in its final extent.

Taking these points into consideration, the electron acceptor most suitable for photoactivation can be characterized by its capability to support a high rate of photoactivation to minimize the photodamage of Mn-depleted complexes and also its capability to protect the photoactivated complexes from photoinactivation. The present study revealed that DCIP at low concentration (5–20 μM) best suffices these requirements as discussed above.

Another finding in this study is an unexpectedly low level of $DCIPH_2$ accumulation during photoactivation. As shown in Fig. 5, $DCIPH_2$ after a photoactivation treatment amounted to only a few micromolar, at most. This may be interpreted in two ways: (i) the PS II complexes in NH_2OH-treated membranes reduce a stoichiometric amount of DCIP on every flash illumination or photoevent by use of Mn^{2+} as electron donor, but most of the reduced DCIP is rapidly reoxidized by Mn^{3+} as previously suggested [12,33], (ii) the quantum yield of DCIP photoreduction during photoactivation is so low by some reason that only a few micromolar DCIP is reduced throughout the whole period of photoactivation. At present we cannot exclude the former interpretation. However, judging from the low quantum efficiency of oxidation of Mn^{2+} by PS II [5] and also from a very low molar ratio of DCIP to PS II reaction center in photoactivation mixture as compared with that optimum for an assay of the Hill reaction, we may assume that many of the PS II complexes are not able to reduce DCIP at every photoevent. Moreover, the finding that the inhibitory effect of higher concentrations of DCIP could be mimicked by reducing only a small portion, 1 μM out of 15 μM DCIP (Fig. 4), supports the latter interpretation. Tamura and Cheniae [11] have proposed that the low quantum efficiency of photoactivation, mainly in terms of formation of the intermediate, results from charge recombination reactions. This implies in turn that the intermediate is stabilized only when the negative charge on the primary or secondary quinone acceptor is transferred to endogenous plastoquinone pool and/or artificial electron acceptors. According to this hypothesis and the two quantum process model for photoactivation [6–8,10], DCIP reduction equimolar to PS II reaction center (1.3 μM in this study) may afford a full recovery, provided the all negative charges are assumed to be transferred to DCIP and reoxidation of $DCIPH_2$ by the intermediate is negligible. Further study is required for a more precise understanding of the role and mode of function of artificial electron acceptors in photoactivation in PS II membranes.

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References

1 Miyao, M. and Murata, N. (1987) in Topics in Photosynthesis, Vol. 9, Photoinhibition (Kyle, D.J., Osmond, C.B. and Arntzen, C.J., eds.), pp. 289–307, Elsevier, Amsterdam.
2 Dismukes, G.C. (1986) Photochem. Photobiol. 43, 99–115.
3 Brudvig, G.W., Beck, W.F. and De Paula, J.C. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 25–46.
4 Amesz, J. (1983) Biochim. Biophys. Acta 726, 1–12.
5 Radmer, R. and Cheniae, G. (1977) in Primary Processes of Photosynthesis (Barber, J., ed.), pp. 303–348, Elsevier, Amsterdam.
6 Radmer, R. and Cheniae, G.M. (1971) Biochim. Biophys. Acta 253, 182–186.

7 Cheniae, G.M. and Martin, I.F. (1973) Photochem. Photobiol. 17, 441–459.
8 Inoue, Y., Kobayashi, Y., Sakamoto, E. and Shibata, K. (1975) Plant Cell Physiol. 16, 327–336.
9 Ono, T.-A., Kajikawa, H. and Inoue, Y. (1986) Plant Physiol. 80, 85–90.
10 Ono, T.-A. and Inoue, Y. (1987) Plant Cell Physiol. 28, 1293–1299.
11 Tamura, N. and Cheniae, G. (1987) Biochim. Biophys. Acta 890, 179–194.
12 Tamura, N. and Cheniae, G.M. (1988) in Light-Energy Transduction in Photosynthesis: Higher Plant and Bacterial Models (Stevens Jr., S.E. and Bryant, D.A., eds.), pp. 227–242, The American Society of Plant Physiologists, Rockville.
13 Yamashita, T. and Tomita, G. (1974) Plant Cell Physiol. 15, 69–82.
14 Yamashita, T. and Tomita, G. (1976) Plant Cell Physiol. 17, 571–582.
15 Ono, T.-A. and Inoue, Y. (1983) Biochim. Biophys. Acta 723, 191–201.
16 Miller, A.-F. and Brudvig, G.W. (1989) Biochemistry 28, 8181–8190.
17 Yamashita, T. and Ashizawa, A. (1985) Arch. Biochem. Biophys. 238, 549–557.
18 Remy, R. (1973) Photochem. Photobiol. 18, 409–416.
19 Oku, T. and Tomita, G. (1976) Physiol. Plant. 38, 181–185.
20 Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87–93.
21 Armstrong, J.M. (1964) Biochim. Biophys. Acta 86, 194–197.
22 Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
23 Ono, T.-A. and Inoue, Y. (1988) Arch. Biochem. Biophys. 264, 82–92.
24 Tamura, N., Inoue, Y. and Cheniae, G.M. (1989) Biochim. Biophys. Acta 976, 173–181.
25 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. USA 78, 7507–7511.
26 Sivaraja, M. and Dismukes, G.C. (1988) Biochemistry 27, 3467–3475.
27 Tamura, N. and Cheniae, G. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 1, pp. 621–624, Martinus Nijhoff, Dordrecht.
28 Vater, J. (1973) Biochim. Biophys. Acta 292, 786–795.
29 Murata, N., Miyao, M., Omata, T., Matsunami, H. and Kuwabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.
30 Klimov, V.V., Shafiev, M.A. and Allakhverdiev, S.I. (1990) Photosynth. Res. 23, 59–65.
31 Becker, D.W., Callahan, F.E. and Cheniae, G.M. (1985) FEBS Lett. 192, 209–214.
32 Kyle, D.J. and Ohad, I. (1986) in Encyclopedia of Plant Physiology New Series, Vol. 19, Photosynthesis III (Staehelin, L.A. and Arntzen, C.J., eds.), pp. 468–475, Springer, New York.
33 Velthuys, B. (1983) in The Oxygen-Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 83–90, Academic Press Japan, Tokyo.