Redox Dependence for Photoligation of Manganese to the Apo-Water-Oxidizing Complex in Chloroplasts and Photosystem II Membranes†

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ABSTRACT: Effects of reducing reagents and redox potentials on photoactivation were studied in Mn-depleted chloroplasts and PSII membranes. Exogenous reducing reagents abolished photoactivation in PSII membranes, while they stimulated photoactivation in chloroplasts. To determine how reducing reagents can have such opposing effects in these preparations, we studied how redox potentials affect photoactivation in the range from 0 mV to +500 mV. In chloroplasts, a modest yield of photoactivation was obtained in the redox potential range of +100 and +330 mV at pH 7.5. The yield of photoactivation decreased at redox potentials above +330 mV, and drastically increased below potentials of +100 mV. Nernst plots of the data show that an $n = 1$ redox component with an $E_{m7.5}$ of +374 mV, as well as an $n = 2$ redox component with an $E_{m7.5}$ of +61 mV, is involved in photoactivation of chloroplasts isolated from dark-grown spruce seedlings. In the case of PSII membranes, photoactivation decreased sharply on either side of +335 mV at pH 5.5. The $n = 1$ redox components with $E_{m5.5}$ of +375 and +319 mV may be involved, both of which showed pH dependences of −60 mV/pH unit. DCMU abolished photoactivation in chloroplasts, but did not affect the dependence of photoactivation on oxidation-reduction potentials in PSII membranes. The component with an $E_{m5.5}$ of +319 mV involved in photoactivation of PSII membranes was also observed in the dependence of Mn solubilization on oxidation−reduction potentials with PSII membranes lacking extrinsic proteins, suggesting that the reduction of Mn with higher valences to Mn(II) by exogenous reducing reagents reversibly occurs in the intermediates or an active center during photoactivation in PSII membranes. Involvement of such redox components in photoactivation in chloroplasts and PSII membranes is discussed.

In higher plants, chloroplasts play a main role in conversion of the light energy to stable chemical forms. The process involves the release of molecular oxygen from water. The reaction of oxygen evolution proceeds via four successive one-electron oxidation steps catalyzed by four Mn atoms, which are located on the oxidizing side of photosystem II (PSII).[1] The manganese is ligated to the PSII reaction center proteins, D1 and D2 (Babcock, 1987; Ghanotakis & Yocum, 1990; Debus, 1992; Renger, 1993). Since photochemical reactions produce strong oxidants in the domain of the reaction center core proteins, particularly the D1 protein, are rapidly photodamaged (Prasil et al., 1992; van Wijk et al., 1994). Consequently, the damaged protein must be replaced by a newly synthesized one, to which manganese atoms are subsequently ligated to form a new water-oxidizing complex.

Ligation of manganese ions to the apo-water-oxidizing complex requires at least two quanta; the process is called photoactivation (or photoreactivation). Photoactivation is thought to include the following steps: (1) binding of Mn^{2+} to the high-affinity Mn binding sites of the apo-WOC; (2) photooxidation of Mn^{2+} after absorption of the first photon; (3) ligation of photogenerated Mn^{3+} in darkness (formation of intermediates of the Mn complex); (4) another ligation of photogenerated Mn^{3+} after absorption of a second photon [formation of the dimer Mn(III)−Mn(III)]; and (5) binding/ligation of another two Mn^{2+} atoms to complete the tetrapolynuclear Mn complex. These steps have been derived from kinetic analysis of photoactivation; however, the molecular basis of this process has yet to be elucidated.

Although a considerable number of papers have been published on photoactivation *in vivo* and *in vitro*, conflicting points remain: concentrations of Mn^{2+}, Ca^{2+}, and Cl^- required for photoactivation (Yamashita & Tomita, 1974; Ono & Inoue, 1983; Tamura & Cheniae, 1987a; Miller & Brudvig, 1989; Miyao & Inoue, 1991), effectiveness of redox reagents (Yamashita & Tomita, 1974; Cheniae & Martin, 1973; Tamura & Cheniae, 1987b; Ono & Inoue, 1987), intactness of membrane permeability (Yamashita, 1982), and involvement of sulfhydryl groups (Oku, 1982) and some factor located on the reducing side of PSI (Ono & Inoue, 1982). Especially confusing has been the role of redox reagents on the yield of photoactivation. Oku and Tomita (1980), with broken chloroplasts from dark-grown spruce seedlings, reported that there is little photoactivation in the

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[1] Abbreviations: Chl, chlorophyll; Cyt *b*-559$_{HP}$, high potential-form of cytochrome *b*-559; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; E_m, midpoint potential; E_h, oxidation−reduction potential; PSII, photosystem II; Q_A and Q_B, primary and secondary plastoquinone electron acceptors; WOC, water-oxidizing complex.

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absence of any reducing reagent, but that photoactivation is remarkably stimulated by addition of reducing reagents such as ascorbate. Yamashita and Tomita (1974) also observed that a certain reducing condition is required for photoactivation of Tris-treated broken chloroplasts that had been depleted of functional Mn. In contrast, with dark-grown *Chlorella* cells (Cheniae & Martin, 1973), intact chloroplasts of wheat seedlings grown under the intermittent light (Ono & Inoue, 1987), and PSII membranes depleted of functional Mn (Tamura & Cheniae, 1987b), such reducing reagents were inhibitory for photoactivation. It is this discrepancy that we sought to explain in this study.

We employed several different types of material for photoactivation: chloroplasts and PSII membranes isolated from dark-grown spruce seedlings, NH_2OH-treated broken chloroplasts from pea seedlings, and NH_2OH-treated PSII membranes from spinach leaves. Dark-grown spruce seedlings have unique characteristics that make them convenient for this study. Although angiosperms form proplastids and etioplasts but no chloroplasts in darkness, the biosynthesis and primary process of greening in conifers such as spruce are not sensitive to light, and thylakoid membranes consisting of light-harvesting chlorophyll proteins and electron transfer components are synthesized (Oku et al., 1975). However, these membranes still lack a functioning oxygen-evolving apparatus, apparently due to a lack of functional Mn. On the other hand, NH_2OH-treated broken chloroplasts from pea seedlings and NH_2OH-treated PSII membranes from spinach leaves represent preparations in which functional Mn and the 17 and 23 kDa extrinsic proteins are artificially removed from membranes. With these preparations, we have studied the effects of various reducing reagents and redox potentials on photoactivation of the water-oxidizing complex and found characteristic redox ranges of photoactivation in chloroplasts and PSII membranes.

MATERIALS AND METHODS

Preparation of Chloroplasts and PSII Membranes. Seeds of spruce [*Picea abies* (L.) Karst] were germinated and grown in vermiculite at 25 °C in complete darkness or under relatively weak light illumination (≈100 μE m^{-2} s^{-1}). After 3 weeks, the needle-like cotyledons were harvested and cut into 1−2-mm length with scissors.

Chloroplasts were prepared from dark- and light-grown cotyledons, respectively, by the procedure of Oku and Tomita (1980) with slight modifications. The cotyledons were homogenized in a preparation buffer [50 mM Tris-HCl (pH 7.8)/0.4 M sucrose/10 mM NaCl/10% (w/v) polyethylene glycol-4000] with a Polytron homogenizer (Kinematica, Sweden), filtered through eight layers of gauze, and centrifuged at 12000*g* for 5 min to precipitate thylakoid membranes. PSII membranes were isolated from the thylakoid membranes as described in Kamachi et al. (1994), with modifications. The spruce thylakoids were solubilized in a resuspending buffer [20 mM Mes−NaOH (pH 6.2)/5 mM $MgCl_2$/15 mM NaCl] containing 2.5% Triton X-100 at 1 mg of Chl mL^{-1}. After 10 min incubation, the suspension was centrifuged at 12000*g* for 3 min, and the supernatant was further centrifuged at 41000*g* for 20 min. The resultant pellet was washed twice in the above buffer to remove an excess of Triton X-100, and the final pellet (PSII membranes) was resuspended in buffer A [50 mM Mes−NaOH (pH 6.5)/0.4 M sucrose/20 mM NaCl] before storage at −80 °C. All procedures were carried out at 4 °C under dim green light. PSII membranes from dark- and light-grown seedlings are described as D-PSII and L-PSII, respectively, in this study.

Broken chloroplasts of pea were isolated from 14-day-old pea seedlings by the procedure of Ikeuchi and Inoue (1986).

Isolation of PSII membranes from market spinach was done as described in Tamura and Cheniae (1987a).

To extract functional Mn from membranes, chloroplasts and PSII membranes (1 mg of Chl mL^{-1}) were treated with 3 mM NH_2OH for 30 min on ice, washed twice with buffer A and employed immediately or after storage at −80 °C.

Photoactivation Procedures. Standard procedure of photoactivation of chloroplasts was as follows: Chloroplasts (250 μg of Chl mL^{-1}) were incubated with TN buffer [20 mM Tricine−NaOH (pH 7.5)/20 mM NaCl] containing 50 μM $MnCl_2$, 5 mM $CaCl_2$, and a reducing reagent, at 25 °C for 30 min under weak light (30 μE m^{-2} s^{-1}) . Photoactivation of PSII membranes was performed as described for chloroplasts, except using buffer A containing 1 mM $MnCl_2$, 50 mM $CaCl_2$, and 100 μM DCIP (Kamachi et al., 1994).

Oxidation−reduction titrations of photoactivation of chloroplasts were done as follows: chloroplasts (50 μg of Chl mL^{-1}) were incubated with TN buffer containing 50 μM $MnCl_2$, 5 mM $CaCl_2$, and redox mediators at 20 μM in a laboratory-made cell (8 mL) for 30 min under weak light (50 μE m^{-2} s^{-1}). The redox mediators used were 2,3,5,6-tetramethylphenylenediamine (E_{m7} = +260 mV), *N*-methylphenazonium methosulfate (E_{m7} = +80 mV), 5-hydroxynaphthoquinone (E_{m7} = +33 mV), *N*-methyl-1-hydroxyphenazonium methosulfate (E_{m7} = −34 mV), 2-hydroxy-1,4-naphthoquinone (E_{m7} = −140 mV), sodium anthraquinone-2-sulfonate (E_{m7} = −225 mV), and benzyl viologen (E_{m7} = −360 mV). The redox potential of the medium was measured with a Pt/Ag−AgCl electrode connected to a pH meter (TOA, Japan). The electrode was calibrated by measuring the E_h of a saturated solution of quinhydrone or ferricyanide/ferrocyanide. The sample was deoxygenated by bubbling with nitrogen gas 5−10 min before a photoactivation procedure. The ambient redox potential was kept constant during photoactivation by addition of potassium ferricyanide or a freshly-prepared dithionite. Following photoactivation, chloroplasts were twice washed with TN buffer and then subjected to measurements for DCIP photoreduction. Redox titrations of photoactivation of PSII membranes were the same, except employing buffer A containing PSII membranes (50 μg of Chl mL^{-1}), 1 mM $MnCl_2$, 25 mM $CaCl_2$, and PSII extrinsic proteins equivalent to approximately 20 nmol, unless otherwise noted.

To obtain E_m and a number (n) of electrons involved in the redox reactions, the dependence of (re-)generated oxygen-evolving activities on E_h was analyzed by a nonlinear curve-fitting Igor program (Wavemetrics, U.S.A.) on a Macintosh computer.

Redox Titrations of Solubilization of Mn in $CaCl_2$-Treated PSII Membranes. $CaCl_2$ treatment was basically done as described in Tamura and Cheniae (1985): Spinach PSII membranes (1 mg of Chl mL^{-1}) were incubated with 0.7 M $CaCl_2$ for 30 min on ice, and then washed twice with buffer A. The obtained preparations proved highly stable (half-time for inactivation by aging of ≈11.5 h) and still retained

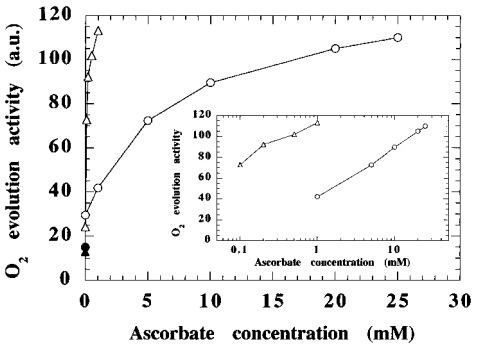

FIGURE 1: Dependence of photoactivation of chloroplasts on ascorbate concentration. Mn-depleted chloroplasts were photoactivated in the presence of a given concentration of ascorbate at pH 7.5. (○) Chloroplasts isolated from dark-grown spruce; (△) pea NH_2OH-treated chloroplasts. Closed symbols represent the oxygen evolution activities of nonphotoactivated chloroplasts.

half the normal amount of the 33 kDa extrinsic protein. Dependence of inactivation on redox potentials in $CaCl_2$-treated PSII membranes was measured as described above for photoactivation of chloroplasts and PSII membranes: The membranes (50 μg of Chl mL^{-1}) were put to a laboratory-made cell with buffer A containing the above mentioned redox mediators (20 μM each) bubbled with nitrogen, and the medium was adjusted to an ambient redox potential by addition of ferricyanide or dithionite. During 30 min incubation on ice, the redox potential of the medium was not observed to be changed. After incubation, aliquots were taken, and oxygen evolution was directly assayed in buffer A containing 1 mM ferricyanide, 500 μM phenyl-*p*-benzoquinone, and PSII extrinsic proteins (mol of proteins/mol of RC $\approx$10).

Other Methods. The DCIP photoreduction was assayed with a Shimadzu UV-300 dual-wavelength spectrophotometer set at 590 nm. The reaction mixture for chloroplasts (10 μg of Chl mL^{-1}) contained a buffer [50 mM Tricine−NaOH (pH 7.5)/20 mM NaCl/0.4 M sucrose] with 50 μM DCIP and 3 mM NH_4Cl, while for PSII membranes (7.5 μg Chl of mL^{-1}) contained buffer A with 50 μM DCIP. O_2-evolving activity was assayed polarographically with a Clark-type electrode described in Tamura and Cheniae (1985). Mn determination was made by an inductively coupled plasma mass spectrometer (Yokogawa PMS-2000) following total digestion of samples.

SDS−PAGE analyses were carried out with a gel system of a 5% stacking and a 12.5% separating polyacrylamide gel containing 5.5 M urea (Ikeuchi & Inoue, 1987). Isolation of PSII extrinsic proteins such as 33, 23, and 17 kDa proteins was done as reported in Tamura and Cheniae (1985).

RESULTS

Effects of Exogenous Reducing Reagents on Photoactivation of Chloroplasts and PSII Membranes Isolated from Dark-Grown Spruce. Photoactivation of the water-oxidizing complex in dark-grown spruce chloroplasts was significantly increased by addition of ascorbate (Figure 1). The concentration of ascorbate needed for half-maximum photoactivation was 3−5 mM, while 20 mM ascorbate proved saturating. The results obtained were the same as previously reported (Oku & Tomita, 1980). We also obtained similar stimulation of photoactivation by ascorbate in NH_2OH-treated chloroplasts in light-grown spruce (data not shown). Photoactivation of NH_2OH-treated chloroplasts from pea seedlings (Figure 1, triangles), however, was over 10 times more sensitive to ascorbate than that of dark-grown spruce chloroplasts. Since we confirmed that added ascorbate stimulates photoactivation in Mn-depleted chloroplasts, we next studied effects of various redox reagents on photoactivation of Mn-depleted chloroplasts and PSII membranes (D-PSII) (Table 1). As shown in Figure 1, there was little restoration of the oxygen evolution activity in chloroplasts after photoactivation treatment without any redox reagent. The addition of all redox reagents employed, except dithionite, increased the yield of photoactivation significantly. On the other hand, a considerable amount of photoactivation of D-PSII was observed even in the absence of redox reagents, as previously reported (Tamura & Cheniae, 1987b). Addition of reducing reagents decreased the yield of photoactivation significantly, independent of the E_m or hydrophobicity of reducing reagents used. These inhibitory effects of added reducing reagents on photoactivation were similar to those with Mn-depleted *Chlorella* cells (Cheniae & Martin, 1973), intact chloroplasts isolated from intermittent-light-grown wheat leaves (Ono & Inoue, 1987), and Mn-depleted spinach PSII membranes (Tamura & Cheniae, 1987b).

Table 1: Effects of Oxidizing and Reducing Reagents on Photoactivation of Chloroplasts and PSII Membranes Isolated from Dark-Grown Spruce Seedlings[a]

redox reagents	chloroplasts	PSII membranes
no addition	18	59
DCIP	87	82
ascorbate	59	31
DCIP + ascorbate	92	48
hydroquinone	37	22
phenazine methosulfate	79	26
ferrocyanide	69	39
dithionite	15	16
nonphotoactivated	10	14

[a] Concentrations of redox reagents were 1 mM, except for DCIP at 100 μM. Photoactivation in the presence of a reagent was carried out under continuous weak light, and DCIP photoreduction was measured as described under Materials and Methods. Photoactivation in chloroplasts and PSII membranes was performed at pH 7.5 and 5.5, respectively.

As shown in the previous study (Tamura & Cheniae, 1987a), photoactivation in PSII membranes was drastically decreased as increasing in concentrations of added ascorbate, which was opposite to the results obtained with chloroplasts (Figure 1). Considering these results, we can exclude the idea that the opposing effect of exogenous reducing reagents on photoactivation in chloroplasts and PSII membranes depends on membrane permeability and results in the difference in concentration of reducing reagents surrounding the Mn-photooxidizing site, probably Tyr_Z. Furthermore, based on the results in Table 1 that a considerable amount of photoactivation in PSII membranes was obtained even in the absence of electron acceptor and that a remarkable yield of photoactivation in chloroplasts was obtained in the presence of reducing reagent but no electron acceptor, it is inferred that rapid electron transfer is not necessarily required for photoactivation in both chloroplasts and PSII membranes. Thus, we suggest that optimal redox atmosphere at both the PSII reducing side and the Mn binding/ligation sites is required for photoactivation of the water-oxidizing complex,

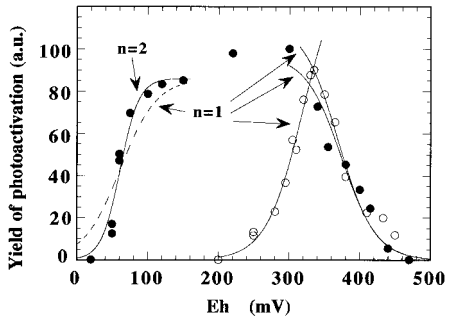

FIGURE 2: E_h dependence of photoactivation in chloroplasts and PSII membranes. Chloroplasts (●) and PSII membranes (○) isolated from dark-grown spruce seedlings were photoactivated at ambient redox potentials. Photoactivation in chloroplasts and PSII membranes was done at pH 7.5 and 5.5, respectively. The $n = 1$ and $n = 2$ Nernst curves are represented by lines.

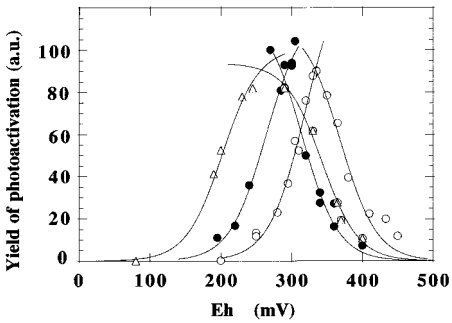

FIGURE 3: pH dependence of redox titrations of photoactivation in PSII membranes. PSII membranes of spruce (circles) and spinach (open triangles) were photoactivated at ambient redox potentials at pH 5.5 and 6.5. (○) pH 5.5; (●) and (△) pH 6.5. The $n = 1$ Nernst curves are represented by lines.

and that there is a remarkable difference in such redox atmosphere between chloroplasts and PSII membranes.

E_h Dependence of Photoactivation in Chloroplasts and PSII Membranes. To learn more about redox regulation of photoactivation, redox titrations of photoactivation were carried out in chloroplasts and PSII membranes. The preparations were anaerobically photoactivated at pH 7.5 and 5.5, respectively. The pH employed was optimal for photoactivation in each preparation. In chloroplasts, photoactivation was maximal at E_h values between +100 and +330 mV (Figure 2). The maximum oxygen evolution activity was comparable to that obtained when chloroplasts were aerobically photoactivated in the presence of appropriate reducing reagents such as ascorbate. The yield of photoactivation dropped off dramatically at E_h >+330 mV and <+100 mV. In the case of D-PSII, no plateau was observed in the E_h dependence of photoactivation, and the optimum E_h for photoactivation was seen as a sharp peak at +335 mV. These data were simulated for an $n = 1$ or $n = 2$ Nernst equation. As shown in Figure 2, data were well fitted to one or the other. In chloroplasts, the E_h dependence below +100 mV was fitted to the $n = 2$ Nernst curve with an $E_{m7.5}$ of +61 mV, while the E_h region above +300 mV was fit to the $n = 1$ curve with an $E_{m7.5}$ of +374 mV. On the other hand, E_h dependences of photoactivation in D-PSII showed $n = 1$ Nernst curves with $E_{m5.5}$'s of +319 and +375 mV in the E_h region below and above +335 mV, respectively. These results are consistent with the difference in reducing reagent effects of photoactivation observed between chloroplasts and PSII membranes in Table 1; that is, the drastic decrease by reducing reagents of photoactivation yield in D-PSII was due to the narrowness of E_h range suitable for photoactivation, and photoactivation in chloroplasts tolerated a more reducing environment than in PSII membranes. The E_h dependence of photoactivation of PSII membranes observed in the range of >+300 mV was similar to that reported by Ananyev and Dismukes (1996), who found that the ratio (1.33) of ferri-/ferrocyanide gave a maximum yield of photoactivation.

Redox titrations of photoactivation in D-PSII were also done at pH 6.5 (Figure 3). The obtained titration curve was very similar to that at pH 5.5, except for a shift of E_h dependence to lower potential by approximately 60 mV. The values of $E_{m6.5}$ were estimated as +263 and +315 mV based on the curve-fitting analysis with the $n = 1$ Nernst equation. We were not able to discern a distinct effect of pH on the E_h dependence of photoactivation in chloroplasts, since the optimal pH for photoactivation was rather narrow.

Figure 3 also shows a similar dependence of photoactivation on E_h between PSII membranes from dark-grown spruce seedlings and NH_2OH-treated spinach PSII membranes from spinach, neither of which possessed functional Mn. In spinach NH_2OH-treated PSII membranes, the higher $E_{m6.5}$ was +343 mV, which was similar to the value (+315 mV) in D-PSII. The lower $E_{m6.5}$ was +202 mV, which is 60 mV lower than the corresponding E_m in D-PSII. These results indicate that the E_h dependence of photoactivation is independent of plant species and of the method utilized for Mn depletion.

Effects of DCMU on Photoactivation in Chloroplasts and PSII Membranes. The decrease in level of photoactivation observed in the E_h range below +100 mV in chloroplasts and above +300 mV in both chloroplasts and D-PSII implies the involvement of endogenous redox components in photoactivation. Judging from E_m values, an oxidized form of an electron transfer component on the reducing side of PSII such as Q_A, Q_B, or bulk plastoquinone is considered to participate in the reaction. Accordingly, we have studied the effects of DCMU on the E_h dependence of photoactivation in chloroplasts and PSII membranes (Figure 4). These preparations were photoactivated in the presence of a given concentration of DCMU, followed by its complete removal by three washes with an excess amount of buffer.

DCMU abolished photoactivation in chloroplasts; the concentration for obtaining the half-maximum inhibition was approximately 0.2 μM (Figure 4A). This DCMU effect was reported with Tris-treated spinach chloroplasts (Yamashita & Tomita, 1974). These results indicate that electron transfer from Q_A to Q_B is required for photoactivation in chloroplasts. Taken together with the results in Figure 2 showing that a redox component with an $E_{m7.5}$ of +61 mV ($n = 2$) is involved, an oxidized state of Q_B is probably required for photoactivation. On the other hand, DCMU did not essentially affect the E_h dependence of photoactivation in D-PSII, although the maximum yield of photoactivation in the presence of 2 μM DCMU was slightly decreased to 95% of that in the absence of DCMU (Figure 4B). The lower $E_{m5.5}$ was estimated as +308 mV, almost identical to that with D-PSII without DCMU, whereas the higher $E_{m5.5}$ of +395 mV was 20 mV greater. We found that, in contrast to chloroplasts, electron transfer from Q_A to Q_B is not required for photoactivation. This result confirmed those previously reported with spinach NH_2OH-treated PSII mem-

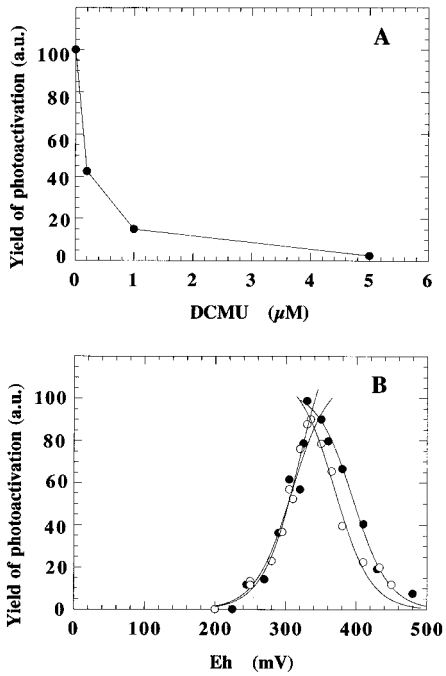

FIGURE 4: Effects of DCMU on photoactivation of chloroplasts and PSII membranes isolated from dark-grown spruce seedlings. Dependence of DCMU concentration on photoactivation of chloroplasts is shown in panel A, while redox titrations of photoactivation in PSII membranes in the presence (●) and absence (○) of 2 μM DCMU are in panel B. Photoactivation in chloroplasts and PSII membranes was done at pH 7.5 and 5.5, respectively. The $n = 1$ Nernst curves are represented by lines.

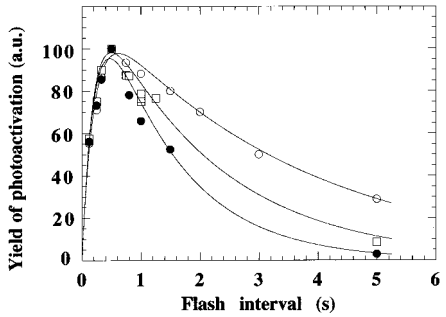

FIGURE 5: Dependence of photoactivation on dark intervals between flashes at different E_h. (●) 190 mV; (○) 250 mV; (□) 380 mV. The photoactivation procedure was done at pH 6.5. The curves are obtained by curve-fittings to the equation described in Tamura and Cheniae (1987a). The data are normalized to yields of photoactivation obtained by flashes with a flash interval of 0.5 s.

branes (Tamura & Cheniae, 1987b). This indicates that the component governing the decline in photoactivation yield in PSII membranes with decreasing E_h is not Q_B, and points to a different mechanism of redox control of photoactivation in chloroplasts and PSII membranes.

Effects of Redox Potentials on Flash-Induced Photoactivation. To clarify which part of the photoactivation process is affected by redox potential, we examined photoactivation induced by 500 flashes at 3 different redox potentials with NH_2OH-treated spinach PSII membranes. This protocol provided information on rate constants for generation and deactivation of the intermediate generated during photoactivation [denoted L_2 in Tamura and Cheniae (1987a)] . As shown in Figure 5, the maximum yield of photoactivation was obtained at the flash interval of 0.5 s, and the yields at +190 and +380 mV were decreased by approximately 50 and 30%, respectively, of the yield at +250 mV. We assumed that an intermediate state is generated (activated) with a rate constant k_A after absorption of one photon and is subsequently deactivated with a rate constant k_D. Curve-fitting led to values of k_A for the intermediate at E_h values of +190, +250, and +380 mV that were 5.20, 4.88, and 4.48 s^{-1}, respectively, while k_D values were 0.502, 0.287, and 0.784 s^{-1}, respectively. The rate constants for activation of the intermediate at +190 and +380 mV were comparable to that obtained at +250 mV, but those for inactivation were significantly greater. As such, these results indicate that rapid deactivation of the intermediate could result in the observed decrease in the yields of photoactivation at +190 and +380 mV. However, it is not clear that acceleration of deactivation of the intermediate at both E_h is due to the same mechanism.

Dependence of Inactivation of the Mn Complex on E_h in PSII Membranes Lacking PSII Extrinsic Proteins. When $CaCl_2$-treated or NaCl-treated PSII membranes were incubated with added reducing reagents, functional Mn atoms were removed from the membranes and the water-oxidizing complex was inactivated, probably resulting from direct reduction of Mn (Ghanotakis et al., 1984; Tamura et al., 1990). Recently, EXAFS and XANES measurements allowed Riggs-Gelasco et al. (1996) to propose that such reducing reagents reduce a site with a lower reduction potential to 'S_{-3}' [Mn(II)Mn(II)−Mn(III)Mn(III)] of the Mn complex and that this state is the same as that of a product generated during the photoactivation process. Thus, we studied the dependence of inactivation of $CaCl_2$-treated PSII membranes on E_h. We employed 0.7 M $CaCl_2$-treated PSII membranes, which were considerably more stable (inactivation by aging at 4 °C with $t_{1/2} \approx 11.5$ h, compared with $t_{1/2} \approx 2.5$ h in 1 M $CaCl_2$-treated ones). The membranes were incubated anaerobically for 30 min at 4 °C at a given E_h in darkness, and O_2 evolution was assayed after reconstitution with PSII extrinsic proteins (see Materials and Methods). The loss of O_2 evolution activity was correlated with the loss of functional Mn from the membranes (data not shown).

Little loss in O_2 evolution capability by 30 min incubation was observed above an E_h of +280 mV. However, upon decreasing the E_h from +280 mV, O_2 evolution was significantly decreased and was completely lost around +100 mV. The data fit well to the $n = 1$ Nernst equation with $E_{m6.5} = +187$ mV, close to that obtained with photoactivation of NH_2OH-treated PSII membranes (+202 mV). Assuming that the redox equilibrium between Mn^{3+} and Mn^{2+} is very rapid in the Mn complex and that the rate-limiting step of an inactivation process is solubilization of Mn^{2+}, these results may indicate that E_m (+187 mV) reflects the midpoint redox potential of Mn(II) and Mn(III) in the Mn complex.

DISCUSSION

We studied the effects of various reducing reagents and redox potentials on photoactivation of different types of Mn-depleted materials. We found the following: (1) the effectiveness of photoactivation is independent of the species of reducing reagent, except dithionite in the case of photoactivation of chloroplasts (Figure 1 and Table 1); (2) photoactivation requires an appropriate range of redox potentials, which are different for chloroplasts and PSII membranes (Figure 2). From these results, it is inferred that the variety of the reported methods for photoactivation result from the requirement of the redox environment which is totally dependent on experimental materials.

Based on redox titrations, photoactivation of chloroplasts from dark-grown spruce seedlings involves redox components with $E_{m7.5}$ values of +374 and +61 mV (Figure 2). In Mn-depleted PSII membranes, redox components with $E_{m5.5}$ values of +375 and +319 mV appear to be involved (Figure 2). Since the higher E_m values were comparable in both types of preparations, a common redox component may function in photoactivation only when this component is in a reduced form. Considering the E_m values, the following electron transfer components are possible candidates: high-potential form of cytochrome *b*-559 (Cyt *b*-559$_{HP}$) and non-heme iron, Q_{400}. The E_m of Cyt *b*-559$_{HP}$ is reported to be +380−+435 mV (Horton et al., 1976; Ortega et al., 1992; Iwasaki et al., 1995), the value of which is independent of pH (Ortega et al., 1992) or shows a slightly positive increase with pH (+60 mV/2.8 pH) (Horton et al., 1976). The E_m of Q_{400} is +370 mV at pH 7.5, which is pH-dependent (−60 mV/pH unit) (Ikegami & Katoh, 1973; Petrouleas & Diner, 1986). To decide between these two candidates, we found the following: (1) the component with D-PSII showed a pH dependence of E_m of approximately −60 mV/pH unit (Figure 3); (2) the proportion of Cyt *b*-559$_{HP}$ was only 11% (0.13 Cyt *b*-559$_{HP}$/PSII) and 46% of the total Cyt *b*-559 in D-PSII and L-PSII, respectively (data not shown); (3) differences in the content of Cyt *b*-559$_{HP}$ among preparations did not affect the yield and rate of photoactivation (data not shown); (4) only a slight increase in E_m by binding of DCMU (Figure 4) was reported in the case of Q_{400} (Wraight, 1985). From these results, the component in question is likely to be Q_{400}, not Cyt *b*-559$_{HP}$. A considerable difference (approximately 100 mV) in E_m values in PSII membranes between Q_{400} and the redox component modifying photoactivation may result from some modifications on the reducing side as well as on the oxidizing side in the Mn-depleted PSII membranes. It is known that the E_m of Q_{400} is drastically decreased by the binding of glycolate (Deligiannakis et al., 1994). However, considering that conversion of a low- or intermediate-potential form to Cyt *b*-559$_{HP}$ occurs simultaneously or immediately before the assembly of the Mn cluster with Tris (pH 9.1)-treated chloroplasts (Mizusawa et al., 1995) and black pines (Shinohara et al., 1992), we cannot exclude the possibility that Cyt *b*-559$_{HP}$ functions in photoactivation in some way. Moreover, if this redox component is Q_{400}, why should the oxidized form of Q_{400} abolish photoactivation in PSII membranes? Tamura and Cheniae (1987a) reported that the addition of DCIP or phenyl-*p*-benzoquinone plus ferricyanide to the photoactivation medium in the presence of atrazine drastically decreased the restoration of the oxygen evolution activity in Mn-depleted PSII membranes. Somehow, electron transfer from Q_A to Q_{400} or an exogenous electron acceptor during photoactivation may cause a decrease in the yield of photoactivation. This question, however, still remains to be answered.

The curve-fitting results in Figure 2 indicated that a two-electron carrier with an $E_{m7.5}$ of +61 mV influences photoactivation and is affected by addition of DCMU (Figure 4). Since E_{m7} values of Q_A/Q_A^- and Q_B/Q_BH_2 have been estimated as −130 and +120 mV, respectively (Diner et al., 1991), we may assign this component as Q_B. Recently, Johnson et al. (1995) reported that a high-potential form of Q_A exists in Mn-depleted PSII, and photoactivation causes the midpoint potential of Q_A to be shifted back from around +55 mV to lower potentials (−80 mV). However, this Q_A is excluded because it is a one-electron carrier. We postulate that redox potentials that fully reduce Q_B increase recombination between Q_A^- and Mn^{3+} in chloroplasts. However, interestingly, this scheme appears not to occur in Mn-depleted PSII membranes since addition of DCMU did not inhibit photoactivation (Figure 4). In PSII membranes, modification of the PSII reducing side may increase the stability of Q_A^- and Mn^{3+}.

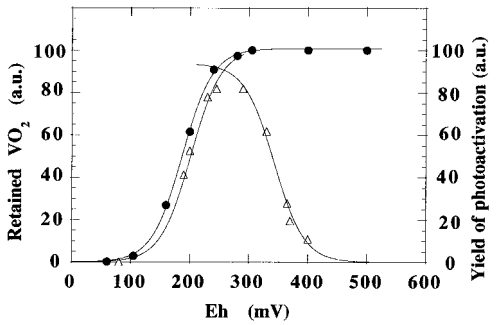

FIGURE 6: E_h dependence of inactivation of the Mn complex in spinach $CaCl_2$-treated PSII membranes. The retained oxygen evolution activities (closed circles) were plotted against given redox potentials, where the membranes were incubated at pH 6.5 for 30 min. Triangles stand for the E_h dependence of photoactivation of PSII membranes, which are presented in Figure 3. The $n = 1$ Nernst curves are represented by lines.

Based on the values of E_m and effects of DCMU on photoactivation, it is clear that the +319 mV component showing an $E_{m6.5}$ of +202 mV in spinach PSII membranes is different from the +61 mV component. There is no known one-electron carrier with such an E_m in PSII, except for intermediate forms of Cyt *b*-559 (Iwasaki et al., 1995). Thus, we studied the E_h dependence of Mn solubilization with 0.7 M $CaCl_2$-treated spinach PSII membranes (Figure 6). The data fit well to the theoretical curve of the $n = 1$ Nernst equation with an $E_{m6.5}$ of +187 mV, which was comparable to the $E_{m6.5}$ of the +202 mV component in spinach PSII membranes (Figure 3). The E_m value of Mn^{2+}/Mn^{3+} in an aqueous solution is known to be very high (+1.51 V in 1 N $HClO_4$); however, Mn^{3+} is stabilized by complexing with EDTA ($E_m = +824$ mV in EDTA and ClO_4^-) (Davies, 1969). The Mn^{2+}/Mn^{3+} ligated with proteins at a sequestered environment may have a much lower E_m. Unfortunately, there are few reports on the E_m values of S-states. Rapid equilibrium between S_1 and S_0 seems to occur in the presence of exogenous reducing reagents such as DCIP and ascorbate (Bouges-Bocquet, 1980). Vass and Styring (1991) reported a midpoint redox potential (≤680 mV) for the S_1/S_0 couple from the equilibria among the S-states and Y_D, the value of which is considerably higher than the value obtained from Figure 6. Interestingly, reports on overreduced forms of the S-states have been accumulated (Messinger et al., 1991; Gleiter et al., 1995; Riggs-Gelasco et al., 1996). Reducing reagents such as redox-active amines can generate the overreduced forms of the S-states like 'S_{-1}' and 'S_{-2}' that are not involved in the normal Joliot−Kok oxygen cycle. Riggs-Gelasco et al. (1996) proposed, based on study of X-ray absorption spectroscopy of the Mn complex in the presence of NH_2OH and hydroquinone, that the complex consists of two Mn dimers with different midpoint redox potentials and that redox-active amines or bulky reducing reagents selectively attack the Mn dimer with lower midpoint redox potential whose oxidation state becomes Mn(II)−Mn(II) in the 'S_{-3}' state. Thus, the E_h dependence of Mn

solubilization observed in $CaCl_2$-treated PSII membranes may reflect the Mn(II)/Mn(III) couple of the binuclear Mn complex in the functional Mn such as the low-potential site postulated by Riggs-Gelasco et al. (1996). Good agreement of the E_h dependence between Mn solubilization in $CaCl_2$-treated membranes and photoactivation of NH_2OH-treated PSII membranes (Figure 6) may lead us to the conclusion that the +202 mV component results from reduction and inactivation of the Mn binuclear that is assembled with the other binuclear [Mn(III)−Mn(III)] at late stages of photoactivation in darkness. As shown in Figure 5, the intermediate generated by absorption of one photon was deactivated 1.8-fold faster at +190 mV than at +250 mV. However, only 40% of the decrease in yield of photoactivation observed at this redox potential can be explained by the acceleration of deactivation of this intermediate. Therefore, we speculate that the decrease in quantum efficiency of photoactivation by the reductive environment results from reduction and solubilization of Mn(III) of an active center and intermediates generated at late stages of photoactivation in a relatively unsequestered domain, concomitantly with those of the intermediates in early stages.

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