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Photoactivation of the water-oxidizing complex of Photosystem II core complex depleted of functional Mn

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Light-induced reassembly (photoactivation) of the water-oxidizing complex was studied using the PS II core complex (TSF2a), which is depleted of functional Mn, PS II extrinsic proteins and light-harvesting chlorophyll proteins. O_2 evolution activity of 300 μmol O_2/mg Chl per h was restored by incubation of TSF2a with a photoactivation medium containing 2 mM $MnCl_2$, 10 mM $CaCl_2$ and the 33/23/17 kDa proteins, under conditions of weak light illumination. The optimum pH for the photoactivation was pH 5.3, that is one pH unit more acidic than for Triton-treated PS II membranes. PS II donor activity and the affinity of Mn^{2+} for the high-affinity Mn^{2+}-binding sites were lower at pH 5.5 than at pH 6.5. The dependence of photoactivation yield using repetitive flashes on the dark intervals between flashes indicated that both the rate constant of generation and rate constant of decay of the unstable intermediate state formed during a photoactivation process drastically decreases with decreases in the pH. These results suggest that lowering the pH leads to destabilization of the intermediate but increases the quantum efficiency of generation of the intermediate.

Introduction

In the oxidizing side of PS II, water is oxidized to oxygen, the reaction of which is catalyzed by the tetra-Mn cluster [1–3]. The water oxidizing complex is considered to be highly integrated with five PS II intrinsic proteins (47 kDa/43 kDa/D1/D2/cyt *b*-559), three PS II extrinsic proteins (33 kDa/23 kDa/17 kDa) and cofactors such as chlorophylls, Fe and quinones. Data from X-ray absorption spectroscopy of PS II membranes [4] showed the spatial configuration of Mn atoms and either N or O atoms seems to be ligated to Mn atoms. The precise identification of the Mn ligation sites was not made. There is evidence [5–7] that functional Mn is ligated to at least the D1 protein, particularly the histidine residues [6,7].

The Mn cluster is destroyed by treatment with NH_2OH, Tris or alkaline pH and is accompanied by release of PS II extrinsic proteins and a loss of O_2 evolution activity. The re-assembly of Mn^{2+} into the apo-complex requires a two-quantum process, demonstrated with variously cultured algae [8], dark-grown gymnosperm leaves [9], intact chloroplasts from leaves greened under intermittent illumination [10], Tris-treated chloroplasts [11] and PS II-enriched membranes treated with $CaCl_2$ plus reducing reagents [12] or NH_2OH [13]. The light-dependent reconstitution (photoactivation) of the apo-complex essentially requires weak light and Mn^{2+}, but neither electrochemical potential across the membranes [14] nor a PS I reducing stroma factor [15]. Cofactors required for advance of the S state such as Ca^{2+}, Cl^- and the 33 kDa protein enhance the efficiency of photoactivation [13,16,17].

To examine the structure and function of the water oxidizing complex in detail, a preparation consisting of a minimum number of proteins and which retains functional Mn has to be used. The O_2-evolving preparation with the smallest size is Ikeuchi's O_2-evolving PS

Abbreviations: TMF2, oxygen-evolving Triton X-100-prepared photosystem II membrane fragments; TSF 2a, Triton subchloroplast fraction 2a; OERC, oxygen-evolving reaction center; DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesufonic acid; DPC, 1,5-diphenylcarbazide; V_{O_2}, rate of O_2 evolution; LHCP, light-harvesting chlorophyll protein.

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II core complex which consists of six proteins (D1/D2/cyt *b*-559/47 kDa/43 kDa/33 kDa) and no light-harvesting chlorophyll proteins (LHCP) [18]. However, it is difficult to further purify the intact preparation made up of less than five proteins as there is a release of functional Mn from membranes by harsh treatment during the isolation procedures. It was reported [19] that photoactivation of the water-oxidizing complex is obtained with the O_2-evolving PS II core complex of which functional Mn is mildly released by NH_2OH treatment. There seems to be no documentation on photoactivation of the PS II core complex and PS II reaction center, preparations which contain neither functional Mn nor PS II extrinsic proteins. We examined photoactivation of the PS II core complex depleted of functional Mn, PS II extrinsic proteins and LHCP and found that the O_2 evolution activity of approx. 300 μmol O_2/mg Chl per h is restored when pH in the photoactivation process is optimized.

Materials and Methods

Triton-extracted PS II membranes (TMF2) from spinach leaves were prepared as described in Ref. 20, resuspended in buffer A (0.4 M sucrose/50 mM Mes-NaOH (pH 6.5)/20 mM NaCl), then used either directly or after storage at $-80\,^\circ$C. Treatment of NH_2OH or Tris with PS II preparations was basically carried out as described in Ref. 13.

O_2-evolving reaction center (OERC) was prepared from wheat seedlings, as described elsewhere [18]. TSF2a from spinach was basically isolated by the procedure given in Refs. 21 and 22 as follows: Chloroplasts were preincubated with Triton X-100 at low concentrations (0.04%) in ST buffer (0.4 M sucrose/50 mM Tris-HCl (pH 7.6)) for 1 h at 4 °C in darkness, centrifuged at 17000 × *g* for 20 min and the pellet was incubated with Triton X-100 at high concentrations (Triton/Chl of 25) at 2.5–3 mg Chl/ml for 1 h at 4 °C in the dark. Then, the suspension was centrifuged at 3600 × *g* for 15 min and the supernatant was ultracentrifuged at 150000 × *g* for 2 h. The resultant pellet was rinsed twice with aliquots of ST buffer and placed in liquid N_2. The frozen membranes were thawed mildly, ultracentrifuged twice at 150000 × *g* for 1 h and the resultant supernatant was used as the TSF2a(sup). To remove excess Triton X-100 from the membranes, TSF2a(sup) was added to a 20-fold vol. of 20 mM Mes-NaOH (pH 6.5) or was loaded onto Bio-beads SM2 and then pelleted by ultracentrifugation at 150000 × *g* for 1 h (TSF2a). TSF2a(sup) and TSF2a showed DCIP photoreduction activities of 1000–1200 and 700–900 μmol DCIP/mg Chl per h, respectively, when diphenylcarbazide (DPC) was used as an electron donor. PS II core complex A was further purified from TSF2a(sup) by an ionic exchange column (DEAE-Toyopearl 650S) chromatography in the presence of 0.01% Triton X-100 at pH 7.2 [23], while the PS II core complex B (B′) purified from photoactivated TSF2a (TSF2a) was subjected to discontinuous sucrose-gradient ultracentrifugation in the presence of 0.1% sucrose monolaurate (SML) at pH 6.5, respectively. As described [23], PS II core complex A showed the DCIP photoreduction activity of 600–800 μmol DCIP/mg Chl per h.

To isolate PS II core complexes B and B′, photoactivated TSF2a and TSF2a, respectively, at 0.5 mg Chl/ml were incubated with 0.5% SML in buffer B (20 mM Mes-NaOH(pH 6.5)/20 mM NaCl) for 1 h at 4 °C. The solubilized membranes (1.5 ml) were layered onto a solution (23 ml) containing 0.1% SML, buffer B and 0.4–1.0 M sucrose discontinuous sucrose gradient and then ultracentrifuged at 150000 × *g* for 4 h with a Hitachi RP50T-2 angle rotor. Two dense green bands were observed at sucrose concentrations at 0.4 and 0.6–0.8 M, respectively. The upper band was a mixture of LHCP and the 43 kDa protein, while the lower band contained a purified PS II core complex. The lower band collected and diluted by 50-fold with buffer B, and concentrated with an Amicon ultrafiltration membrane (YM-30). Isolated PS II core complexes B and B′ showed the DCIP photoreduction activity of 300–400 μmol DCIP/mg Chl per h.

Photoactivation of various types of preparation was done under weak continuous light (25–30 μE/m^2 per s), essentially as described elsewhere [13]. DCIP photoreduction was measured spectrophotometrically at 590 nm on the split mode with a Hitachi-557 spectrophotometer. DCIP photoreduction by Mn^{2+} (1 mM) or diphenylcarbazide (500 μM) was basically measured in a reaction mixture containing buffer A, 50 μM DCIP and TSF2a (5 μg Chl/ml) or Tris-TMF2 (10 μg Chl/ml). O_2 evolution was determined polarographically as described in Ref. 13: The reaction mixture contained buffer A, 250 μM phenyl-*p*-benzoquinone, 1 mM ferricyanide, 10 mM $CaCl_2$, $CaCl_2$ extracts (30 μg protein/ml) and PS II preparations equivalent to 7.5 μg Chl/ml, unless otherwise noted. Mn was determined as described in Ref. 23. SDS-polyacrylamide gel electrophoresis analyses were carried out as described in Ref. 6. $CaCl_2$ extracts were prepared as described [12].

Results

Photoactivation of various types of PS II preparation

Fig. 1 shows polypeptide compositions of TMF2, Tris-TMF2, TSF2a(sup), TSF2a, and PS II core complexes A and B, respectively. The polypeptide composition of TSF2a(sup) was similar to that of the O_2-evolving PS II core complex, except for a small amount of LHCP and 10–25 kDa proteins (Lane 3). TSF2a, ob-

tained by diluting and centrifuging TSF2a(sup), was constituted of mainly five proteins (47 kDa/43 kDa/D1/D2/cyt *b*-559) but contained little 33 kDa protein (Lane 4). The Mn content of the preparation was 0.2 Mn/40 Chl and no O_2 evolution activity was observed. PS II core complex A, further purified with an ionic exchange column chromatography, lacked most of the LHCP and several minor bands in the molecular mass range of 10–25 kDa (Lane 5). In the gel, there was an unidentified 55 kDa protein. PS II core complex B, which was purified from photoactivated TSF2a with discontinuous sucrose gradient ultracentrifugation, also lost LHCP, several minor polypeptides in the molecular mass range of 10–25 kDa and an unidentified 55 kDa protein (Lane 6). Part of the 43 kDa protein was solubilized during the isolation procedure. The polypeptide composition of PS II core complex B′ was the same as that of the PS II core complex B (data not shown). A small amount of the 33 kDa protein detected in the PS II core complex B was probably due to the rebinding of the 33 kDa protein to membranes during the photoactivation of TSF2a. Low-molecular-mass proteins such as psb F, psb I and psb K were detected in all PS II preparations used in this work (data not shown).

Table I shows the photoactivation capacity in PS II membranes and PS II core complexes. The preparations (250 μg Chl/ml or 100 μg Chl/ml) were photoactivated by incubating under weak light at 23°C for 45 min in buffer A containing 1 mM $MnCl_2$, 50 mM $CaCl_2$ and 100 μM DCIP and $CaCl_2$ extracts (30 μg/ml), as described elsewhere [13]. When the NH_2OH-treated O_2-evolving reaction center (OERC) was incubated under these conditions the regenerated activity of O_2 evolution reached 300 μmol O_2/mg Chl per h (line 3), values close to those obtained with NH_2OH- and Tris-TMF2 [13]. NH_2OH-OERC mainly consists of 47 kDa/43 kDa/D1/D2/cyt *b*-559 and the

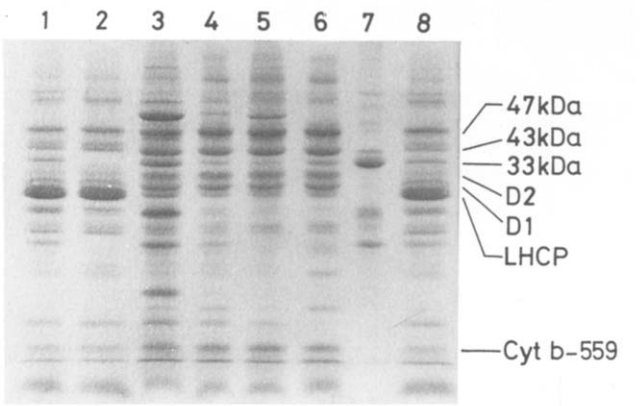


Fig. 1. SDS-polyacrylamide electrophoresis of PS II membranes and PS II core complexes. Lanes 1 and 8, TMF2; Lane 2, Tris-TMF2; Lane 3, TSF2a(sup); Lane 4, TSF2a; Lane 5, PS II core complex A; Lane 6, PS II core complex B; Lane 7, $CaCl_2$ extracts.

TABLE I

Photoactivation capacity in various types of PS II preparation

Photoactivation was done as described in Materials and Methods, and O_2 evolution activity was assayed at pH 6.5. The chlorophyll concentrations used for NH_2OH- or Tris-TMF2 was 250 μg Chl/ml and those for PS II core complexes were 100 μg Chl/ml. Values of regenerated activities of O_2 evolution in NH_2OH- and Tris-TMF2 were referred from data reported in Ref. 13. Numbers in parentheses are O_2 evolution activities of preparations which were photoactivated at pH 5.5. TMF2 and OERC showed O_2 evolution activities of 590 and 910 μmol O_2/mg Chl per h, respectively. There was no O_2 evolution activity in TSF2a(sup), TSF2a or PS II core complexes

Preparations	Regenerated V_{O_2} (μmol O_2/mg Chl per h)
1. NH_2OH-TMF2	380
2. Tris-TMF2	290
3. NH_2OH-OERC	310
4. TSF2a(sup)	0 (0)
5. TSF2a	125 (310)
6. PS II core complex A	0 (17)
7. PS II core complex B	12 (58)
8. PS II core complex B′	7 (20)

33 kDa protein, as we reported [6]. Kaloska and Cheniae also reported the photoactivation of the NH_2OH-treated, O_2-evolving PS II core complex [19]. The materials which we all used for photoactivation, including NH_2OH-OERC, are O_2-inactive PS II preparations obtained by NH_2OH- or Tris-treatment from O_2 active PS II ones that contain functional manganese and PS II extrinsic proteins. Whenever purified PS II core complexes with less than five proteins are prepared, functional Mn is removed from the water-oxidizing complex. To explore minimum units of the water-oxidizing complex, the PS II core complex or the PS II reaction center should be reconstituted with exogenous Mn^{2+}. Photoactivation of TSF2a(sup) did not occur under the condition examined (line 4). When the residual Triton X-100 was removed from TSF2a(sup) by loading on Bio-beads SM2 or by diluting and subsequently ultracentrifuging, a considerable amount (310 μmol O_2/mg Chl per h) of the O_2 evolution activity was restored. As shown in Fig. 1, TSF2a was constituted of mainly five proteins (47 kDa/43 kDa/D1/D2/cyt *b*-559) but functional Mn and PS II extrinsic proteins were removed during the isolation procedures. When we examined photoactivation of PS II core complexes further purified from TSF2a, little O_2 evolution activity was regenerated by photoactivation of the PS II core complex A or B′ at pH 5.5 (line 6 or 8). The O_2 evolution activity of 50–60 μmol O_2/mg Chl per h was restored by photoactivation of PS II core complex B (line 7), obtained from photoactivated TSF2a.

These results indicate that several minor polypeptides in the molecular mass range of 10–25 kDa and LHCP are not essential for the photoligation of

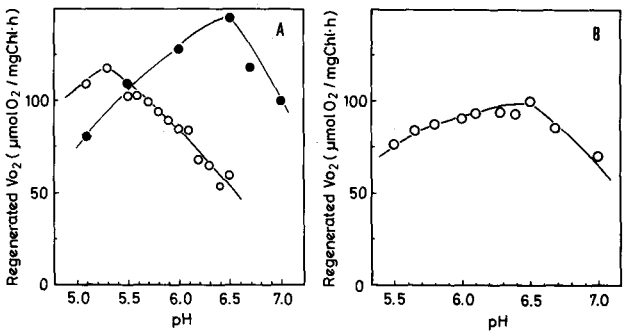


Fig. 2. pH dependencies of photoactivation of TSF2a (A) and expression of O_2 evolution in photoactivated TSF2a (B). In (A), TSF2a.(○) or Tris-TMF2 (●) was photoactivated at various pH values and O_2 evolution activity was assayed at pH 6.5. In (B), TSF2a was photoactivated at pH 5.5 and O_2 evolution activity was assayed at various pH values.

Mn^{2+} to the apo-complex. These observations also suggest that regeneration of the Mn cluster in TSF2a aids in avoiding denaturation of the Mn-binding site during further purification in the presence of detergents such as Triton X-100 and SML.

Characteristics of photoactivation of TSF2a

Fig. 2A shows the dependencies on pH of photoactivation of TSF2a and Tris-TMF2. The optimum for photoactivation of Tris-TMF2 was obtained at pH 6.5. This pH profile was similar to those for NH_2OH-TMF2 [13] and $DCIPH_2$-treated $CaCl_2$-TMF2 [12], but is in contrast to the optimum at pH 7.8 for Tris-treated chloroplasts [24]. On the other hand, the regenerated activity in O_2 evolution with TSF2a increased with decreases in the pH. The weak pH optimum for the photoactivation of TSF2a was 5.3. The ratio of O_2 evolution activity for TSF2a photoactivated at pH 5.5 to that for TSF2a photoactivated at pH 6.5 varied from 1.5 to 4 in different batches of TSF2a. The dependence on assay pH of the O_2 evolution activity was studied in TSF2a photoactivated at pH 5.5 (Fig. 2B). The optimum for the O_2 evolution expressed by the photoactivated TSF2a was obtained at pH 6.5. The same value was noted with NH_2OH-TMF2 [13]. These results suggest that the regenerated Mn complex of TSF2a functions in water oxidizing activity in the same manner as that of NH_2OH-TMF2. The kinetics of photoligation of Mn^{2+} to the apo-complex of TSF2a probably differs from that of NH_2OH-TMF2.

To determine why photoactivation of TSF2a proceeds at pH 5.5 more efficiently than at pH 6.5, we studied the pH dependence of DCIP photoreduction donated by Mn^{2+} or diphenylcarbazide (DPC) (Fig. 3). The concentrations, K_m, giving the half-maximal rates were obtained between pH 5.3 and pH 6.5. When Mn^{2+} was used as the artificial PS II donor, the K_m value increased with a decrease in the pH: The value (167 μM) at pH 5.5 was about 6-fold greater than that at pH 6.5. The K_m values were essentially independent of the pH when DPC was used as a PS II electron donor. The rate obtained at a saturating concentration of a PS II electron donor, V_{max}, was plotted against the pH in Fig. 3B. The pH dependence of V_{max} was similar to that of K_m. The V_{max} values with DPC slightly increased with a decrease in the pH, whereas values with Mn^{2+} were drastically decreased with a decrease in the pH. Thus, the efficiency of an electron donation of Mn^{2+} to Z^+ or $P680^+$ was lower at pH 5.5.

We also examined pH dependence of the affinity of Mn^{2+} to the high-affinity Mn binding sites, using competitive inhibition by exogenous Mn^{2+} of DPC-supported DCIP photoreduction [25]. The inhibition constant, K_i, of TSF2a at pH 6.5 was 1.0 μM, that is approx. 10-times larger than that of Tris-TMF2 (data not shown). The obtained value with Tris-TMF2 was similar to the value noted with Tris-treated PS II membranes [26]. The K_i value of TSF2a drastically increased with a decrease in the pH. These results suggest that the affinity of the exogenous Mn^{2+} to the apo-complex becomes weaker at pH 5.5.

Fig. 4A shows the dependence of photoactivation of TSF2a at pH 5.5 on $MnCl_2$ concentration. In the presence of 50 mM $CaCl_2$, the half-maximal and maximal yield of photoactivation for TSF2a were obtained at 1 and 2 mM $MnCl_2$, respectively. The values obtained were much larger than those reported with NH_2OH-TMF2 [13]. The Mn^{2+} concentration optimum for photoactivation depended on the $CaCl_2$ concentration used for photoactivation (Fig. 3B). The optimum concentration of $CaCl_2$ for photoactivation of

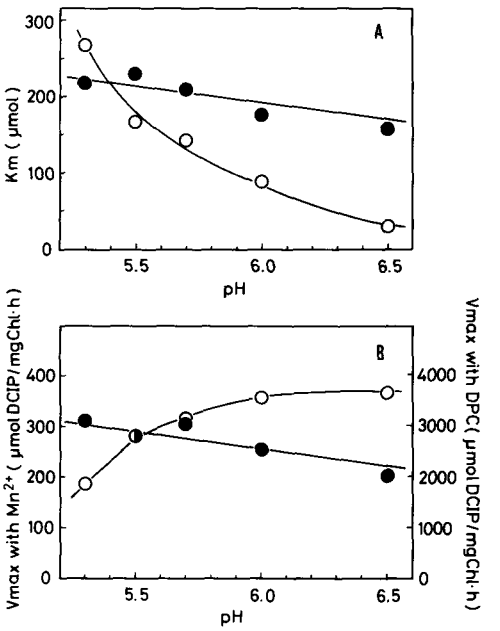


Fig. 3. pH dependencies of K_m (A) and V_{max} (B) for DCIP photoreduction of TSF2a. $MnCl_2$ (○) or DPC (●) was used as an exogenous PS II electron donor.

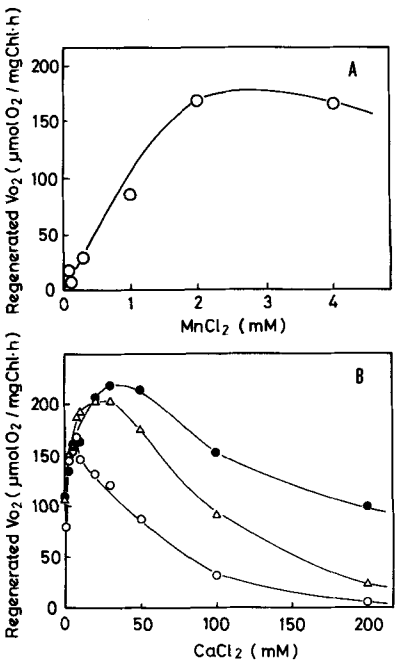


Fig. 4. Dependence of photoactivation of TSF2a on $MnCl_2$ (A) and $CaCl_2$ concentrations. Photoactivation of TSF2a was done at pH 5.5. The concentration of $CaCl_2$ for photoactivation was fixed at 50 mM in (A). In (B), the concentration of $MnCl_2$ was fixed at 0.5 (○), 2 (△) or 4 (●) mM.

TSF2a was shifted from 7.5 to 30 mM with increasing Mn concentrations from 0.5 to 4 mM. When TSF2a was photoactivated with 1 mM $MnCl_2$ at pH 6.5, the $CaCl_2$ concentration optimum for photoactivation was 50 mM (data not shown). The yield of photoactivation at pH 5.5 with 0.5 mM $MnCl_2$ decreased to 3.5% of the maximum yield in the presence of 200 mM $CaCl_2$, while the yield with 4 mM $MnCl_2$ retained about half the maximum yield. These results support the previously stated conclusions [13,17] that the competition between Mn^{2+} and Ca^{2+} occurs at binding sites for these cations in the water-oxidizing complex.

Since the yield of photoactivation of TSF2a at pH 5.5 was much greater than that at pH 6.5, we expected that the affinity of Mn^{2+} to the Mn-ligation sites would become stronger at pH 5.5 than at pH 6.5. However, results in Figs. 3 and 4 show that photooxidation of Mn^{2+} at pH 5.5 is poor, and that the affinity of Mn^{2+} to the high affinity Mn binding sites becomes weaker with a decrease in the pH.

Time-courses of photoactivation of TSF2a under conditions of continuous weak light illumination were examined at pH 6.5 and 5.5 (data not shown). As previously shown [13], a photoactivation process followed the first-order kinetics. The time required for the half-maximum yield of photoactivation was 7–8 and 18–20 min at pH 6.5 and 5.5, respectively. These results suggest that the first-order rate constant of the limiting step in the photoactivation process is smaller at pH 5.5.

To analyze the effect of pH on the kinetics of photoactivation in detail, effects of pH on photoactivation by repetitive flashes separated by different dark intervals (t_d) were studied (Fig. 5). The regenerated O_2 evolution activity was almost proportional to the flash number when the flash numbers were varied from 50 to 500. The O_2 evolution activity regenerated by 500 flashes is considered to be a measure of the relative quantum efficiency of the various flash regimes for the photoconversion of an inactive Mn complex to an active one. At both pH, the dependencies of O_2 evolution activities on dark intervals between flashes showed typical bell-shaped curves, as previously reported [13,27–29]. The half-time for the generation and decay of the intermediate at pH 6.5 was 1 s and 2.6 s, respectively. These values were much larger than those obtained with NH_2OH-TMF2 [13]. On the other hand, at pH 5.5, the half-time for the ascending and descending portions was 2.6 s and 3.5 s, respectively. According to the previously proposed mechanisms involving two quantum processes [8,13], the ascending and descending portions of the curve reflect the generation and decay, respectively, of the unstable intermediate in a photoactivation process (see Discussion). These results indicate that both processes of generation and decay of the intermediate of TSF2a are modified even at pH 6.5.

We then examined the dependence on pH of stability of Mn^{3+} generated by continuous light, using the indirect method. When DCIP and exogenous Mn^{2+} are used as the electron acceptor and the electron donor respectively, there was a light-induced DCIP photoreduction and subsequent reoxidation of $DCIPH_2$ on turning off the light illumination, as shown in Fig. 6A. The reoxidation of $DCIPH_2$ is probably due to the recombination of $DCIPH_2$ and intermediate (Mn^{3+}?)

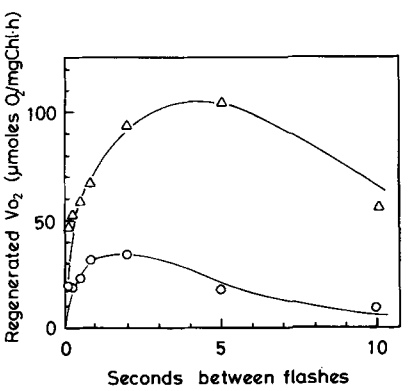


Fig. 5. Dependence of photoactivation of TSF2a by repetitive flashes on dark time between flashes. TSF2a was illuminated with 500 saturated flashes (under 5 μs) in the photoactivation medium as described in text. Curves are obtained by the following equation (Eqn. 2 in Ref. 13); $[L_2]_n = \phi(k_A/(k_D - k_A))(e^{-k_A t_d} - e^{-k_D t_d})$.

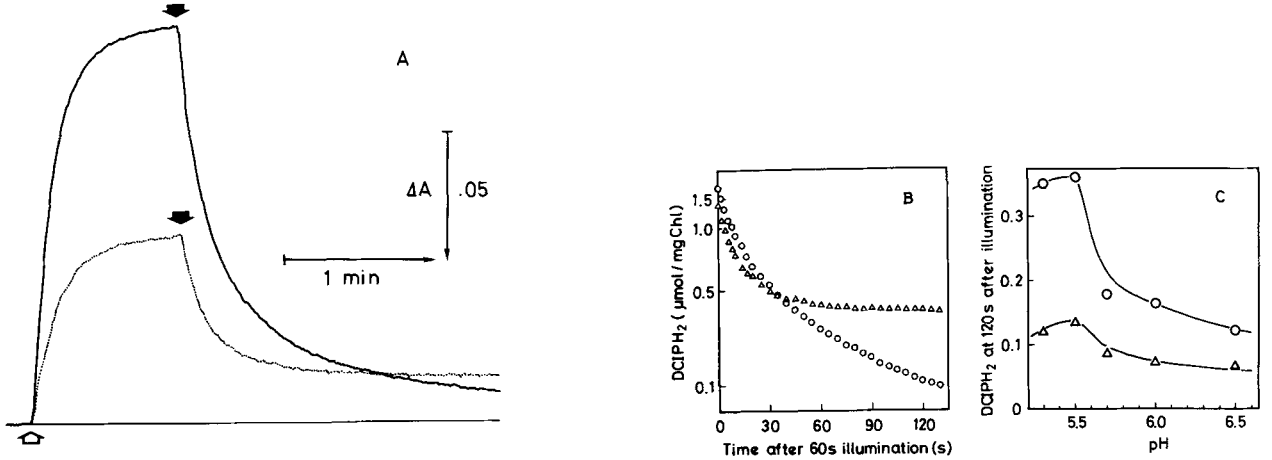


Fig. 6. DCIP photoreduction and the subsequent $DCIPH_2$-reoxidation in darkness when 1 mM $MnCl_2$ was used as the electron donor. Time-courses of light-induced DCIP reduction and the following $DCIPH_2$ reoxidation on turning off 1 min illumination at pH 5.5 (dotted line) and 6.5 (continuous line) are shown in A. The amounts of $DCIPH_2$ at the steady state during the illumination were 1.40 and 1.73 μmol DCIP/mg Chl at pH 5.5 and 6.5, using extinction coefficients for DCIP of 10.6 and 18.0 mM^{-1} cm^{-1}, respectively. (B) Semilogarithmic plots of $DCIPH_2$ reoxidation at pH 5.5 (Δ) and 6.5 (○). (C) pH dependence of the amount of $DCIPH_2$ at 120 s after turning off the light. TSF2a (○); Tris-TMF2, (△).

generated during the illumination [30–32]. Within 1 min, the DCIP photoreduction reached a steady state level, which was almost independent of pH. The reoxidation of $DCIPH_2$ was biphasic (Fig. 6B): The fast phase with $t_{1/2}$ of approx. 7 s was independent of pH, whereas the half time of the slow phase increased from 57 to > 500 s with a decrease in the pH from pH 6.5 to 5.5. The amount of $DCIPH_2$ at 120 s after turning off the light was about 3-fold larger at pH 5.5 than at pH 6.5 (Fig. 6C). A similar pH dependence was obtained with Tris-TMF2. These results imply that a part of intermediate (Mn^{3+}?) generated by the illumination becomes less reactive $DCIPH_2$ at the lower pH. There may be an increase in more stabilized Mn^{3+} in the microenvironment of the Mn-binding sites at the lower pH.

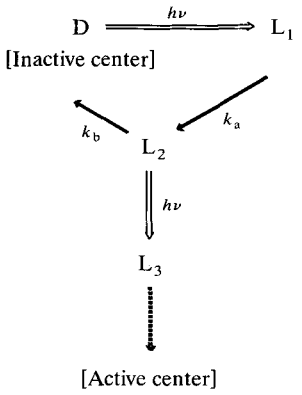
Discussion

We examined photoactivation of TSF2a which lost functional manganese and PS II extrinsic proteins during the isolation procedure. We succeeded in photoactivating TSF2a, only when the residual Triton X-100 was ultimately removed by loading on Bio-beads SM2 or diluting and subsequently ultracentrifuging (Table I, line 5). Interestingly, the pH optimum for the photoactivation of this preparation was around 5.3, that is one pH unit more acidic than that for the photoactivation of NH_2OH-TMF2 (Fig. 2A). The pH optimum for expression of the regenerated center was to be 6.5 (Fig. 2B), thereby indicating that the regenerated Mn cluster of TSF2a functions to advance the S state in the same manner as the intact one of PS II membranes.

The shift of pH optimum for the photoactivation was expected to result from changes in factors such as the photooxidation of Mn^{2+}, the affinity of Mn^{2+} to high-affinity Mn-binding sites and the binding of the 33 kDa protein. However, the capacity of the Mn^{2+}-photooxidation at pH 5.5 was inferior to that at pH 6.5 (Fig. 3). The high-affinity Mn-binding sites seem to be further modified at pH 5.5, as deduced from the following results: (i) The value of K_m of Mn^{2+} in the photoactivation was much higher at pH 5.5 than at pH 6.5 (Fig. 3); (ii) The competitive inhibition constant (K_i) of Mn^{2+} in DPC-supported DCIP photoreduction, the constant of which is considered to be a constant of dissociation of Mn^{2+} from the high-affinity binding sites, was much greater at pH 5.5 than that at pH 6.5; (iii) Ca^{2+} at lower concentrations competitively inhibited the photoactivation of TSF2a at pH 5.5 (Fig. 4B). Furthermore, there was no difference in the capacity of binding of 33 kDa protein between pH 6.5 and pH 5.5 (data not shown). Thus, one cannot explain the unique pH dependence of photoactivation of TSF2a using these factors only.

We focused on the kinetics of generation of the intermediate in the photoactivation process. When we studied the time-course of the photoactivation of TSF2a at pH 5.5 and pH 6.5, the yield of the photoactivation was lower at pH 6.5 than at pH 5.5, but the generation of the active center was approx. 3-times faster at pH 6.5 than at pH 5.5. This may be explained by the following: The ratio of rate constant of decay to that of generation of the intermediate becomes smaller at pH 5.5 than at pH 6.5 and the rate constants at pH 5.5 are smaller than those at pH 6.5. Alternatively, the quan-

tum efficiency of the photoactivation may increase as the pH decreases. The photoactivation of the apo-water oxidizing complex by a flash illumination provides the two-quantum series model for photoactivation [8,13]:



Where L_1, L_2 and L_3 are the intermediates formed during photoactivation, and k_a and k_b are rate constants for the generation of L_2 from L_1 and the decay of L_2 to D, respectively. When we used Eqn. 2 in Ref. 13, we were able to get the theoretical curves well fitted to the experimental data (Fig. 5). Rate constants for the generation and decay of the intermediate (L_2) were much smaller at pH 5.5 than at pH 6.5: The values for k_a and k_b were obtained at 0.27 s^{-1} and 0.20 s^{-1} at pH 5.5, while 0.69 s^{-1} and 0.27 s^{-1} at pH 6.5, respectively. To fit the rapid phase of the ascending portion, we assumed that 15% of the total centers obeyed the same kinetics as we observed in photoactivation of NH_2OH-TMF2 (k_a and k_b, 5.5 s^{-1} and 0.87 s^{-1}, respectively). The ratio of k_a to k_b was approx. 1.5-fold larger at pH 6.5 than at pH 5.5. Taking these values and assuming that the quantum efficiency (ϕ in equation 2 in Ref. 13) for photoactivation is the same at both pH, we expected that the yield of L_2 would be 1.65-fold greater at pH 6.5 than at pH 5.5. As this notion contrasts to the results in Fig. 5, we suggest that the higher yield of photoactivation of TSF2a at pH 5.5 is due to a 4-fold increase of the quantum efficiency of the photoactivation, probably resulting from an increase in yield of the ligation of Mn^{3+} photooxidized to the apo-complex in a process of L_1 to L_2 and/or a process of L_3 to the active center.

It remains to be determined why the ligation of Mn^{3+} to the apo-complex efficiently occurs at pH 5.5. The stability of photogenerated species such as Mn^{3+} and Q_A^- or Q_B^- may be required for photoactivation of the apo-complex. We previously reported effects of various artificial electron acceptors on the yield of photoactivation of NH_2OH-TMF2 [13]. We found: (i) that an electron acceptor which is good for higher electron transfer rates, such as phenyl-*p*-benzoquinone, was not adequate for photoactivation, (ii) about half the maximum yield of photoactivation was obtained even when no electron acceptor was added or when electron transfer between Q_A and Q_B was blocked by atrazine, and (iii) in the presence of atrazine, addition of DCIP drastically decreased the yield of photoactivation. These results suggest that rapid electron transfer is not necessarily required for photoactivation and that photoactivation requires an adequate life time of Mn^{3+} and Q_A^- or Q_B^-. At present, the stabilization by acidic pH of Mn^{3+} photooxidized may be referred to the results in Fig. 6. DCIP photoreduction by exogenous Mn^{2+} with TSF2a or Tris-TMF2 showed that the reduced DCIP was reoxidized rapidly in subsequent darkness, probably via the recombination of intermediate (Mn^{3+}?) and the reduced DCIP [30–32]. Based on this phenomena, the effect of pH on the recombination between reduced DCIP and Mn^{3+} was examined. The extent and rate of reoxidation of the reduced DCIP were gradually suppressed when decreasing the assay pH to an acidic one. This result indicates that the photogenerated Mn^{3+} becomes less reactive with $DCIPH_2$ near the Mn-ligation sites. This leads to the idea that the recombination between Mn^{3+} and intrinsic PS II secondary acceptors, Q_A^- and Q_B^- is slower at acidic pH, the result being an increase in quantum efficiency of the ligation of Mn^{3+} to the Mn-ligation sites in the apo-water oxidizing complex, regardless of a decrease in stability of the intermediate.

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References

1 Amesz, J. (1983) Biochim. Biophys. Acta 726, 1–12.
2 Dismukes, G.C. (1986) Photochem. Photobiol. 43, 99–115.
3 Babcock, G.T. (1987) in New Comprehensive Biochemistry, Photosynthesis (Amesz, J., ed.), pp. 125–152, Elsevier, Amsterdam.
4 Yachandra, V.K., Guiles, R.D., McDermott, A., Britt, R.D., Dexheimer, S.L., Sauer, K. and Klein M.P. (1986) Biochim. Biophys. Acta 850, 324–332.
5 Taylor, M.A., Nixon, P.J., Todd, C.M., Barber, J. and Bowyer, J.R. (1988) FEBS Lett. 235, 109–116.
6 Tamura, N., Ikeuchi, M. and Inoue, Y. (1989) Biochim. Biophys. Acta 973, 281–289.
7 Seibert, M., Tamura, N. and Inoue, Y. (1989) Biochim. Biophys. Acta 974, 185–191.
8 Radmer, R. and Cheniae, G.M. (1977) in Primary Process of Photosynthesis (Barber, J., ed.), Vol. 2, pp. 301–348, Elsevier, Amsterdam.
9 Oku, T. and Tomita, G. (1976) Physiol. Plant. 38, 181–185.
10 Ono, T. and Inoue, Y. (1983) Biochim. Biophys. Acta 723, 191–201.

11 Yamashita, T. and Tomita, G. (1976) Plant Cell Physiol. 17, 571–582.
12 Tamura, N. and Cheniae, G.M. (1986) FEBS Lett. 200, 231–236.
13 Tamura, N. and Cheniae, G.M. (1987) Biochim. Biophys. Acta 890, 179–194.
14 Yamashita, T. (1982) Plant Cell Physiol. 23, 833–841.
15 Ono, T.-A. and Inoue, Y. (1982) Plant Physiol. 69, 1418–1422.
16 Yamashita, T. and Ashizawa, A. (1985) Arch. Biochem. Biophys. 238, 549–557.
17 Tamura, N., Inoue, Y. and Cheniae, G.M. (1989) Biochim. Biophys. Acta 976, 173–181.
18 Ikeuchi, M. and Inoue, Y. (1986) Arch. Biochem. Biophys. 247, 97–107.
19 Kalosaka, K. and Cheniae, G.M. (1988) Plant Physiol. 86, 131.
20 Radmer, R., Cammarata, K., Tamura, N., Ollinger, O. and Cheniae, G.M. (1986) Biochim. Biophys. Acta 850, 21–32.
21 Vernon, L.P. and Shaw, E.R. (1971) Methods Enzymol. 23, 277–288.
22 Ke, B., Vernon, L.P. and Chaney, T. (1972) Biochim. Biophys. Acta 256, 345–357.
23 Inoue, H. and Wada, T. (1987) Plant Cell Physiol. 28, 767–773.
24 Yamashita, T. and Ashizawa, A. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Murata, N., Crofts, A.R., Renger, G., Govindjee and Satoh, K., eds.), pp. 327–336, Academic Press Japan, Tokyo.
25 Hsu, B.-B., Lee, J.-Y. and Pan, R.-L. (1987) Biochim. Biophys. Acta 890, 89–96.
26 Klimov, V.V., Allakhberdiev, S.V., Shuvalov, V.A. and Krasnovsky, A.A. (1982) FEBS Lett. 148, 307–312.
27 Cheniae, G.M. and Martin, I.F. (1971) Biochim. Biophys. Acta 253, 161–181.
28 Inoue, Y., Kobayashi, Y., Sakamoto, E. and Shibata, K. (1975) Plant Cell Physiol. 16, 327–336.
29 Yamashita, T., Inoue, Y., Kobayashi, Y. and Shibata, K. (1978) Plant Cell Physiol. 19, 895–900.
30 Izawa, S. (1970) Biochim. Biophys. Acta 197, 328–331.
31 Velthuys, B. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Murata, N., Crofts, A.R., Renger, G., Govindjee and Satoh, K., eds.), pp. 83–90, Academic Press Japan, Tokyo.
32 Tamura, N. and Cheniae, G.M. (1988) in Light-Energy Transduction in Photosynthesis: Higher Plants and Bacterial Models (Stevens, Jr., S.E. and Bryant, D.A., eds.) pp. 227–242, The American Society of Plant Physiologists.