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Removal of Ca by pH 3.0 treatment inhibits S_2 to S_3 transition in photosynthetic oxygen evolution system

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Effects of Ca extraction by pH 3.0 treatment on the electron transport on donor side of Photosystem II (PS II) were investigated. The flash O_2 yield was abolished by the treatment, and was restored to normal by the addition of Ca^{2+} with almost no change in the oscillation pattern. The light-induced fluorescence increase was lost by the treatment and was well restored by addition of Ca^{2+}. Among the other cations tested, only Sr^{2+} was effective. The treated membranes exhibited a light-dependent EPR Signal II_{fast} superimposed on dark-stable Signal II_{slow}, but the Signal II_{fast} was lost on addition of Ca^{2+}, suggesting a Ca-dependent reversible conversion between Signal $II_{very\ fast}$ and Signal II_{fast}. The peak temperatures of thermoluminescence B- and Q-bands arising from $S_2Q_B^-$ and $S_2Q_A^-$ charge recombinations, respectively, were elevated to high temperatures after the treatment, and were largely reversed to their respective normal temperatures by the addition of Ca^{2+}. When excited by a series of flashes, the amplitude of the modified B-band generated by the 1st flash did not change any more after the 2nd flash. These results were interpreted as indicating that extraction of one of two Ca from higher plant PS II by pH 3.0 treatment induces an abnormal S_2 state and thereby inhibits S_2 to S_3 transition.

Introduction

Ca has first been proposed to be a cofactor for cyanobacterial O_2 evolution based on the observation that the maximum rate of O_2 evolution by cyanobacterial membranes requires exogenous Ca^{2+} [1]. In cyanobacteria, the Ca requirement is demonstrated readily after washing the PS II particles with EGTA or a Ca-free buffer medium [2,3], and becomes more pronounced in purified O_2 evolving PS II particles [2] or in cells grown under Ca-deficient conditions [3,4]. Ca is required for electron transport from DPC to DCIP [5] as well as for reduction of P-680$^+$ in the submicrosecond time range [6]. These findings are interpreted as suggesting that Ca is an essential cofactor in the electron transport from Z to P-680$^+$.

Later, a requirement for Ca in higher plant PS II reactions was suggested by Barr et al. [7] which based on the effects of EGTA- or acid treatments on O_2 evolution, although the results were somewhat complicated by non-specific background cation effects. The involvement of Ca in higher plant PS II reactions has been shown more clearly by the observation that Ca is indispensable for photoactivation of the latent O_2-evolving activity in chloroplasts grown under intermittent flashes [8,9] or during photoreactivation of O_2 evolution in Tris- or NH_2OH-treated chloroplasts or isolated PS II membranes [10,11]. More distinct stimulation by Ca of O_2 evolution has been reported for NaCl-washed PS II membranes depleted of the 16 and 24 kDa extrinsic proteins [12,13], for $CaCl_2$-washed membranes depleted of the 16, 24 and 33 kDa extrinsic proteins [14], or for detergent-solubilized LHCP-free O_2-evolving PS II complex [15]. In contrast to the observations with cyanobacterial membranes, the addition of Ca to NaCl-washed membranes of higher plants accelerated the decay of Signal II_{fast} (Z^+) [16,17], suggesting that Ca is essential for the electron transport from the Mn cluster to Z. Involvement of Ca in the S-state transition has also

Abbreviations: PS, Photosystem; Q_A, primary quinone acceptor of Photosystem II; Q_B, secondary quinone acceptor of Photosystem II; Z, secondary donor of Photosystem II; DPC, 1,5-diphenylcarbazide; DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Chl, chlorophyll; D, auxiliary secondary donor of Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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been proposed based on the inhibitory and restoring effects of NaCl-wash and Ca addition, respectively, on the flash number dependence pattern of 2 ms luminescence [18] or thermoluminescence [19]. However, the site(s) of Ca action in these inactivation and reactivation is not yet clarified. The absence of Ca-dependent O_2 evolution in normal PS II membranes has been attributed to a contribution by the 24 kDa extrinsic protein which hinders the light-triggered liberation of Ca from PS II compelx [20]. This view, however, is not always consistent with the observation that rather a large part of PS II centers remain functional in O_2 evolution even after complete removal of the 16 and 24 kDa extrinsic proteins by NaCl-wash [19,21]. These situations have made it difficult to understand the functional site of Ca in higher plant PS II.

Recently, we developed a new technique for discrete extraction of Ca from higher plant PS II membranes, in which we treated the membranes at pH 3.0 for a short period [22]. This treatment extracted in darkness only one out of the two Ca atoms per PS II reaction center, concomitant with nearly 90% loss in O_2 evolution but with no loss of any of the extrinsic proteins, and the treated membranes restored a high rate of O_2 evolution when supplemented with exogenously added Ca. In the present study, we report the Ca-dependent properties of the electron transport on donor side of PS II in pH 3.0 treated membranes and discuss the functional site of this low pH sensitive Ca in the O_2-evolving reaction.

Materials and Methods

Triton X-100-solubilized PS II membranes capable of O_2 evolution were prepared from spinach as described in Ref. 19, and stored in liquid N_2. After thawing, the PS II membranes were washed twice with 400 mM sucrose, 20 mM NaCl, 0.1 mM Mes-NaOH (pH 6.5) and resuspended in the same medium. The membranes were then treated with 400 mM sucrose, 20 mM NaCl, 20 mM citrate-NaOH (pH 3.0) at a chlorophyll concentration of 2 mg Chl per ml. After 5 min incubation at 0 °C in darkness, the treated membranes were diluted with 400 mM sucrose, 20 mM NaCl and 40 mM Hepes-NaOH (pH 7.5) to give a final pH of about 7.5, and then subjected to measurements of electron transport and thermoluminescence after dilution with the same buffer (pH 7.5) medium. When indicated, the treated membranes were suspended in 0.4 M sucrose, 20 mM NaCl, 40 mM Mes-NaOH (pH 6.5). For EPR measurements the treated membranes were centrifuged at $35000 \times g$ for 10 min, and the resulting pellet was resuspended in the same buffer (pH 7.5) medium.

Flash O_2 yield was measured in the absence of artificial electron acceptor with a Joliot-type O_2-rate electrode under a series of Xe flashes (white, 4 μs; 2 J) given at a uniform interval of 1 s as described in Ref. 19. Chl *a* fluorescence transient was measured at room temperature as in Ref. 14. O_2 evolution was measured at 25 °C with a Clark-type oxygen electrode with 0.8 mM phenyl-*p*-benzoquinone as an electron acceptor as described in Ref. 19. EPR spectra were recorded at 15 °C with a JEOL X-band EPR spectrometer, model JES FE1XG. For thermoluminescence measurements, dark adapted samples were excited with a saturating Xe flash (white, 4 μs, 2 J), cooled rapidly in liquid N_2, and the light emission during warming the sample (approx. 1 °C per s) was recorded against sample temperature as described previously [19].

Results

Fig. 1 shows the effect of pH 3.0 treatment on flash O_2 yield. Untreated PS II membranes showed a typical period-4 oscillation with maxima at the 3rd and 7th flashes (open circles). However, the oscillation was appreciably damped from the second cycle on, and the signal amplitude was strongly diminished after more than 10 flashes. This is mainly due to the limited number of plastoquinone molecules as acceptor pool. (Note that no artificial electron acceptor was employed.) In pH 3.0 treated membranes, the flash O_2 yield was totally suppressed except for a very faint signal (open squares), but retained an almost normal oscillation pattern with maxima at the 3rd and 7th flashes when expressed in expanded scale. This is probably due to the contribution by non-inhibited centers remaining after the treatment. On addition of 50 mM $CaCl_2$ to the treated membranes, the O_2 signal suppressed by the treatment was fully restored (solid circles). As opposed to the reconstitution of O_2 evolution so far reported for $CaCl_2$-washed [19] and NaCl-washed [23] membranes, the reconstituted flash O_2 pattern of the pH 3.0 treated membranes exhibited no distortion at all. These results

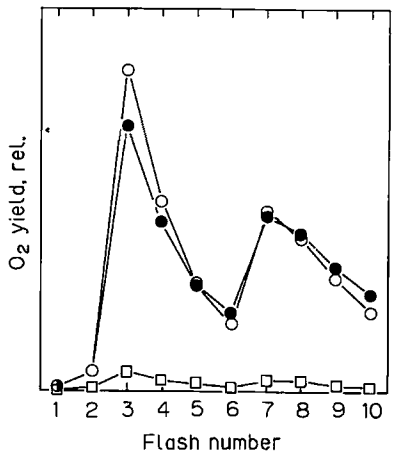

Fig. 1. Effect of pH 3.0 treatment on flash O_2 yield pattern. Control PS II membranes (○), pH 3.0 treated membranes (□), and pH 3.0 treated membranes after 30 min dark incubation at 0 °C with 50 mM $CaCl_2$ (●).

TABLE I

Comparison of Ca^{2+} effect on O_2-evolving activity in pH 3.0 treated and NaCl-washed PS II membranes under flash and continuous illuminations. For flash illumination, the figures in parentheses represent the average values (with standard deviation, ±S.D.) of the flash O_2 yield at the 3rd flash with no electron acceptor added. For continuous illumination, the figures in parentheses represent O_2 evolution in μmol O_2 per mg Chl per h. The electron acceptor was 0.8 mM phenyl-*p*-benzoquinone. Light (≧ 420 nm) intensities for both flash and continuous illuminations were saturating.

Sample	Addition	O_2 evolution (%)	
		flash illumination	continuous illumination
Control	None [a]	100 (12.8±0.84)	100 (540)
	50 mM $CaCl_2$	126 (16.2±0.54)	107 (580)
pH 3.0 treated	none	7 (1.00±0.12)	13 (75)
	50 mM $CaCl_2$ [b]	104 (13.4±0.51)	92 (500)
NaCl-washed	none	90 (11.6±0.57)	65 (350)

[a] Basal reaction medium contained 20 mM NaCl.

[b] O_2 evolution was measured after 30 min incubation at 0°C with $CaCl_2$.

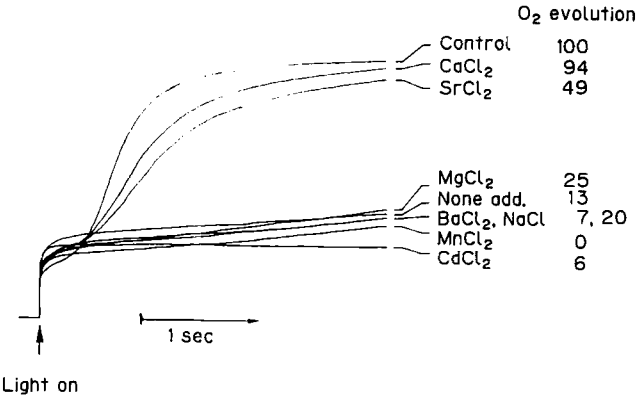

Fig. 2. Effects of various cations on fluorescence transient and O_2 evolution in pH 3.0 treated membranes. Treated membranes were supplemented with 50 mM (100 mM for NaCl) of various cations (chloride salt), and fluorescence transient and O_2 evolution were measured after 30 min dark incubation at 0°C. O_2-evolving activity of the untreated membranes was 650 μmol per mg Chl per h with 0.8 mM phenyl-*p*-benzoquinone as acceptor, and the activities of treated membranes in the presence of respective divalent cations added are indicated in percent. Measurements with untreated control membranes were done in the presence of 50 mM $CaCl_2$.

indicate that pH 3.0 treatment effects an all-or-none type inactivation of O_2 evolution, which can be attributed to extraction of Ca from the O_2-evolving centers.

Table I compares the effect of pH 3.0 treatment on the rate of O_2 evolution under flash and continuous illumination. The extents of activity inhibition by pH 3.0 treatment and activity restoration by Ca^{2+} addition determined under the two illumination protocols correlated well with each other. The activities under flash illumination were estimated from the relative O_2 yield after the 3rd flash on the profiles indicated in Fig. 1. These data are largely compatible with the Ca-dependent stimulation of O_2 evolution reported for NaCl-washed PS II membranes depleted of 16, 24 kDa extrinsic proteins [12,13,19], but differ in the following point: In NaCl-washed PS II membranes, the loss in O_2 evolution under flash illumination (10%) is much less than that under continuous illumination (35–50%), which is presumably due to the light-dependent release of Ca in NaCl-washed PS II [17,19,20]: incomplete release after dark NaCl-wash is completed under continuous illumination during O_2 measurement but not under flashes for flash O_2 yield measurement. Addition of Ca^{2+} to control membranes slightly enhanced the activity, which may be due to partial loss of 16 and 24 kDa extrinsic proteins that would have occurred during preparation and/or storage in liquid N_2.

Fig. 2 shows the effects of pH 3.0 treatment on PS II electron transport activities as measured by O_2 evolution and fluorescence transient. The fluorescence transients of the treated membranes were devoid of variable fluorescence, although they showed the same F_0 level as that in control membranes. This indicates that pH 3.0 treatment inhibits the electron transport on the donor

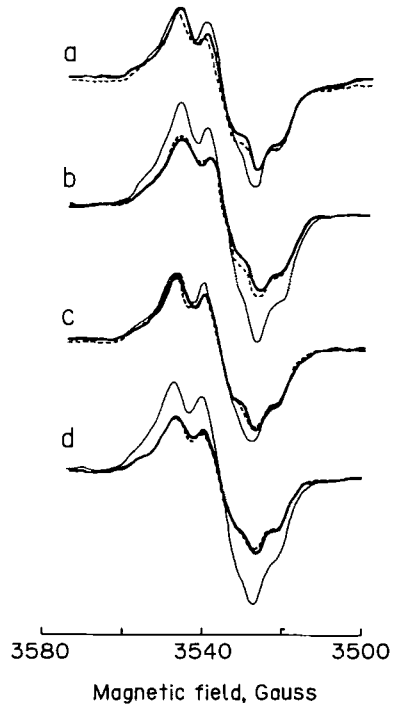

Fig. 3. Effect of pH 3.0 treatment on EPR Signal II_{slow} and Signal II_{fast}. Control membranes (a); pH 3.0 treated membranes (b); pH 3.0 treated membranes after incubation with 50 mM $CaCl_2$ at 0°C for 30 min (c); and 0.8 M Tris (pH 9.3) treated membranes (d). Bold curves indicate the spectra in the dark, thin curves the spectra during illumination and dotted curves the spectra in the dark after illumination followed by 5 min dark incubation. Chl concentration was 3 mg Chl per ml. Instrumental settings: microwave power, 5 mW; modulation frequency and amplitude, 100 kHz and 4 G, respectively; scan rate, 25 G/min; temperature, 15°C.

side of PS II. This is in agreement with our previous finding that the electron transport from DPC to DCIP is not affected by the treatment in spite of complete inhibition of O_2 evolution [22]. On addition of Ca^{2+} to the treated membranes, fluorescence variation was markedly restored concomitant with about 90% restoration in O_2 evolution. The restoration effect was pronounced with Ca^{2+}, appreciable with Sr^{2+} but negligible with Na^+, Mg^{2+}, Ba^{2+}, Cd^{2+} and Mn^{2+} in both fluorescence transient and O_2 evolution. In control membranes, addition of these cations did not affect fluorescence transient, except for Cd^{2+} which suppressed the transient (data not shown).

The effect of pH 3.0 treatment on PS II donor side electron transport was studied by measuring EPR Signal II. As depicted in Fig. 3, untreated membranes showed only Signal II_{slow} both in the light and dark (curve a), which arises from D^+, an auxiliary electron donor of PS II. In pH 3.0 treated membranes, light-dependent Signal II_{fast} which arises from Z^+, the oxidized form of the secondary electron donor of PS II, was observed during illumination being superimposed on dark-stable Signal II_{slow} (curve b). These EPR spectra were identical with those of Tris-inactivated PS II membranes (curve d), indicating that re-reduction of Z^+ is suppressed in pH 3.0 treated membranes. When 50 mM $CaCl_2$ was employed to the treated membranes, the light-dependent Signal II_{fast} was specifically suppressed, leaving the dark-stable Signal II_{slow} unaffected (curve c). This suggests that Signal II_{fast} was converted back to Signal $II_{very\ fast}$ [24] by exogenous Ca^{2+}. From these results, it was concluded that extraction of Ca by pH 3.0 treatment does not affect the electron transport from Z to P-680, but interrupts the electron transport to Z^+ from the water-oxidizing complex. All these results are in agreement with the Ca-induced restoration of O_2 evolution and fluorescence variation in pH 3.0 treated membranes shown in Figs. 1 and 2.

The effect of pH 3.0 treatment on the electron transport in PS II donor side was further studied by means of thermoluminescence. As depicted in Fig. 4, a single flash excitation of untreated PS II membranes yielded the B-band at around 35°C which arises from $S_2Q_B^-$ charge recombination (c). When Q_A to Q_B electron transport was blocked by DCMU, the B-band was replaced by the Q-band at 13°C, which arises from $S_2Q_A^-$ charge recombination (f). In contrast, the pH 3.0 treated membranes showed an abnormal upshifted sharp band at around 45°C both in the presence and absence of DCMU (b and e). On addition of 50 mM $CaCl_2$, the abnormal thermoluminescence bands with upshifted peak temperatures were reversed back to normal bands with respective normal peak temperatures, although the restored B-band ($S_2Q_B^-$) was slightly broadened (a), and the restored Q-band ($S_2Q_A^-$) retained an appreciable shoulder at a higher temperature (d). In view of the fact

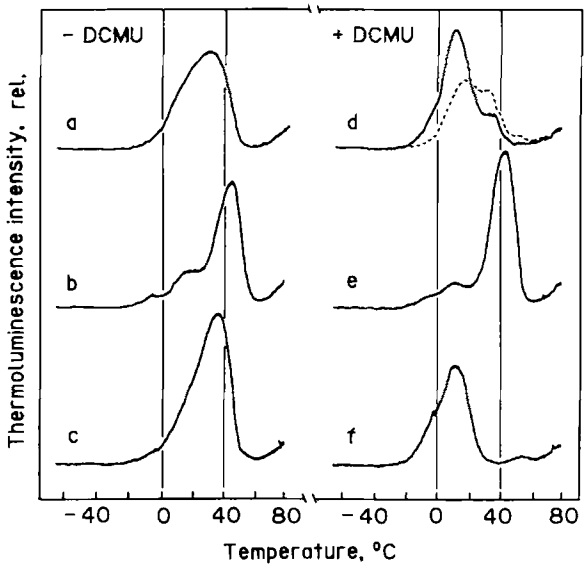

Fig. 4. Effect of pH 3.0 treatment on thermoluminescence glow curves excited by one flash at 0°C in the absence ($S_2Q_B^-$ charge pair, left panel) and presence of 10 μM DCMU ($S_2Q_A^-$ charge pair, right panel). Control membranes, (c and f); pH 3.0 treated membranes (b and e); and pH 3.0 treated membranes after incubation with 50 mM $CaCl_2$ at 0°C for 30 min (a and d). The broken glow curve was obtained by post-flash $CaCl_2$ addition, in which 50 mM $CaCl_2$ was added to the pH 3.0 treated sample immediately after flash excitation, and thermoluminescence was measured after additional dark incubation for 30 s at 0°C.

that the emission of thermoluminescence B- and Q-bands requires a storage of positive charge on the water-oxidizing Mn cluster [25], these results are interpreted as indicating that the pH 3.0 treated PS II centers are still capable of accumulating an oxidizing equivalent in the O_2-evolving complex as an S_2 state, although its redox properties are appreciably modified as revealed by the upshifted peak temperature of B- and Q-bands. By analogy with the abnormal $S_2Q_B^-$ and $S_2Q_A^-$ charge pairs reported to be induced by Cl^--depletion [26], the parallel upshift of the peak temperature of both the B- and Q-bands suggests that the pH 3.0 treatment affects these two charge pairs by preferentially modifying the redox properties of S_2 but not those of Q_B^- or Q_A^-.

The dotted curve (d) in Fig. 4 is the glow curve obtained by a post-flash Ca^{2+} addition protocol, in which 50 mM $CaCl_2$ was added to the treated membranes in darkness immediately after flash excitation followed by incubation at 0°C for 30 s before the sample was cooled in liquid N_2. This protocol resulted in a glow curve showing a peak at around 15°C accompanied with a distinct shoulder at around 30°C. This indicates that Ca-dependent restoration of the redox properties of the S-state can proceed in S_2 state as well as in S_1 state. The abnormal high peak temperature (45°C) was shifted back to a lower normal temperature (15°C), but the resulting glow curve is considerably

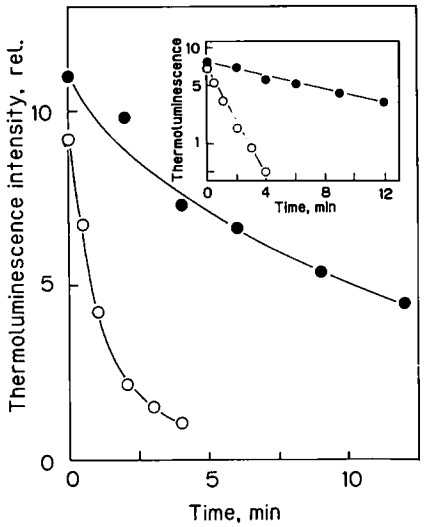

Fig. 5. Effect of pH 3.0 treatment on the stability of $S_2Q_B^-$ charge pair. Control (○) and pH 3.0 treated (●) membranes. Samples were illuminated with one flash and cooled to 77 K after varied period of dark incubation at 25 °C. The amplitude of the glow curve was plotted against the time of 25 °C dark incubation. The inset shows a semilogarithmic plot of the decay course.

different from the normal Q-band ($S_2Q_A^-$) still showing a high temperature component. This is in accordance with our previous finding that the course of reconstitution of functional Ca (as expressed by acquirement of EDTA-resistant O_2 evolution as an index) is a slow process [22]. Judging from the fact that the same shoulder is observed on the normally reconstituted glow curve (solid curve d), the shoulder component may be attributed either to an intermediate state during ligation of Ca^{2+} to acquire EDTA-resistance or to a partial damage of the Mn cluster during the treatment. It may be of note in this context that the reconstituted B-band ($S_2Q_B^-$) is much broader (a) than the control B-band (c).

Fig. 5 shows the decay kinetics of the $S_2Q_B^-$ charge pair created in pH 3.0 treated membranes. The amplitude of $S_2Q_B^-$ band showed a monophasic decay with an approximate half-time of 1 and 8 min at 25 °C in control and pH 3.0 treated membranes, respectively. The result as well as the upshifted peak temperature of both B- and Q-bands imply that the pH 3.0 treatment results in an increased stability of $S_2Q_B^-$ pair, e.g., a deeper trap of S_2 due to a lowered redox potential by analogy with the abnormal S_2 in Cl^--depleted PS II [26,27].

Fig. 6A shows the feature of S-state turnovers in pH 3.0 treated membranes. Well dark-adapted PS II membranes were illuminated with series of flashes at 10 °C, and the oscillatory behavior of flash-induced glow peak was investigated. In untreated membranes (left panel), the 1st flash generated a glow peak at around 40 °C due to $S_2Q_B^-$ charge recombination in the major (approx. 63%) centers, which were in S_1Q_B state in the initial dark adapted conditions (see also Fig. 4). After the 2nd flash, a little less intense glow peak was found at around 35 °C. According to the theory of thermoluminescence oscillation [28], this peak is due to superposition of two contributions by $S_3Q_B^-$ (approx. 13%) and $S_2Q_B^-$ (approx. 13%), which were in $S_1Q_B^-$ and $S_0Q_B^-$, respectively, in the initial dark-adapted state. The downward shift of peak temperature after the 2nd flash is indicative of formation of $S_3Q_B^-$ charge pair which is known to recombine at a lower temperature than $S_2Q_B^-$ charge pair. Note that the pH of the suspending medium was lowered to 6.5 to facilitate the separation between $S_3Q_B^-$ and $S_2Q_B^-$ bands [27]. After the 3rd flash, the intensity of glow peak markedly declined, indicating that most of the centers were converted to S_1 and S_0 states (having no positive charge) after three times of PS II photoreactions. In pH 3.0 treated membranes (right panel), the 1st flash generated a glow curve having unusually high peak temperature at around 45 °C (see also Fig. 4). However, this glow peak did not show any change in intensity and peak temperature after the 2nd and 3rd flashes, and kept the same glow curve as observed after the 1st flash.

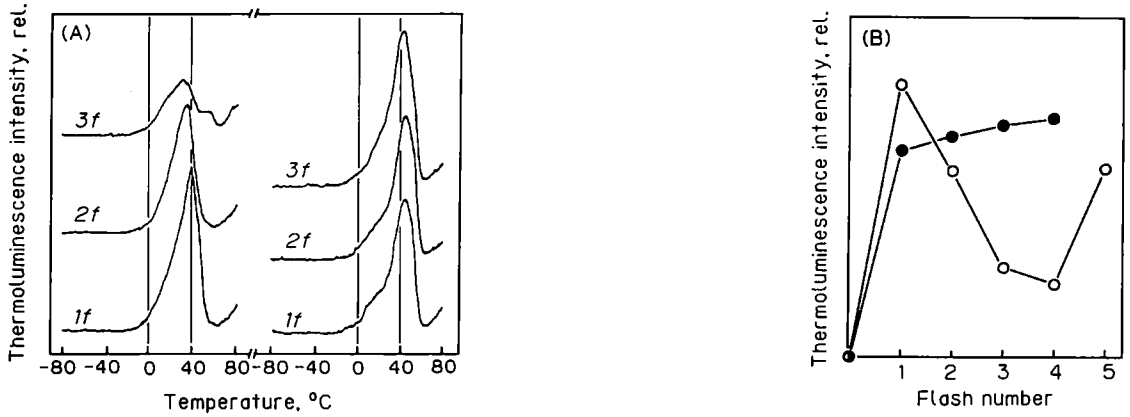

Fig. 6. Effect of pH 3.0 treatment on the thermoluminescence oscillation. (A) Oscillatory behavior of glow curves during the first three flashes in untreated (left panel) and pH 3.0 treated (right panel) PS II membranes. (B) Oscillation pattern of total luminescence during the first five flashes in untreated (○) and treated (●) PS II membranes.

Fig. 6B compares these oscillatory behaviors measured during the first five flashes, in which the total luminescence, the integrated area under the glow peak shown in Fig. 6A, was plotted as a function of flash number. The control membranes (open circles) showed a typical quadruple oscillation of flash 1,5 pattern [19,26,28] as predicted from the initial distribution of $S_1 : S_0 = Q_B : Q_B^- = 75 : 25$. In pH 3.0 treated membranes (closed circles), however, the oscillation was interrupted after the 1st flash, showing only a gradual increase in intensity with increase in flash number. The gradual increase may be attributable to the centers (25%) relaxed in S_0 state in the initial dark-adapted condition and/or the center retained as S_1 due to misshit on the 1st flash. These results indicate that in pH 3.0 treated PS II S-state turnover does not proceed beyond S_2 state.

Discussion

In our previous paper [22], we have shown that pH 3.0 treatment removes one out of the two Ca atoms associated with a unit of PS II and thereby inactivates O_2 evolution. Our thermoluminescence data in the present study (Figs. 4–6) revealed that the treatment induces an unusually upshifted glow peak and interruptes S_2 to S_3 transition. These data well account for the EPR and fluorescence data (Figs. 2 and 3). By analogy with the similarly abnormal glow peaks found in Cl^--depleted [26,27] or NH_3-binding [29] PS II, the unusual glow peak is attributed to modulation of the thermodynamic properties of $S_2Q_B^-$ charge pair, lowering of the oxidation potential of S_2 as a result of the removal (one of the two) of Ca.

As to the Ca-depletion induced by NaCl-wash, two alternative inhibition sites have been proposed: inhibition of S_1 to S_2 transition deduced from the loss of EPR multiline signal [30,31], and inhibition of S_3 to S_0 transition from the interruption of flash-dependent oscillation of delayed fluorescence [18], thermoluminescence [19] and EPR multiline signal [32]. This confusion may be related to limited information about Ca abundance in sample PS II : No Ca determination has been done in some of the reports. Some papers report that NaCl-wash decreases Ca abundance to one Ca per reaction center and inactivates O_2 evolution by 50–70%, but the inactivation extent increases to 90% without further removal of Ca when chelator and ionophore are included during the wash [21], while another paper reports the NaCl-wash inactivates O_2 evolution by 60–70% without affecting the Ca abundance (2 Ca per PS II) [33]. These discrepancies may have arisen from the situations that Ca release by NaCl-wash is a light-dependent process [16,19,20], and NaCl-wash inevitably involves release of the 24 kDa extrinsic protein which is known to stabilize Ca-binding [20,34]. Under these circumstances, it is difficult at present to give a comprehensive view about the Ca-depletion in NaCl-washed PS II.

Admitting these divergencies in interpretation of the Ca-related phenomena in NaCl-washed PS II, our results in the present study apparently are incompatible with those so far reported for the Ca-depletion induced by NaCl-wash. The major differences are: (i) interruption of S_2 to S_3 transition after pH 3.0 treatment vs. interruption of S_1 to S_2 [30,31] or S_3 to S_0 [18,19,32] after NaCl-wash; (ii) extrinsic proteins (24 and 16 kDa) are all retained in the former [19] vs. mostly lost in the latter [12,13,20,34]; (iii) marked Ca-demand is observed after treatment in darkness [19] vs. only after treatment under illumination [16,19,20]; (iv) restoration of O_2 evolution requires high Ca concentration (approx. 40 mM) [19] vs. saturates at low concentration (approx. 5 mM) [12,13,20,34].

Cyanobacterial PS II is reported to contain only one Ca, whose removal results in inhibition of Z to P-680$^+$ electron transport [35]. For higher plant PS II, however, no treatments have been confirmed to remove more than one Ca per a unit of PS II, although many Ca-dependent activity restorations have been reported. If we assume that the number of Ca atoms in PS II is two [21–23], most of the inhibitions reported as caused by Ca-removal may be ascribed to the loss (or modulation) of one of the two Ca atoms, probably the weakly bound one. Presumably, the tightly bound Ca is functional in supporting the Z to P-680$^+$ electron transport, whereas the other weakly bound Ca functions in facilitating the electron transport in water cleavage reactions, and its removal induces variously modulated S-state transitions. Taking these into account, the effect of NaCl-wash and pH 3.0 treatment on PS II may be tentatively characterized as follows: pH 3.0 treatment completely releases the weakly bound Ca in darkness, while NaCl-wash just loosens in darkness or partially releases the same weakly bound Ca under illumination.

As to the location in PS II of the Ca-binding site susceptible to pH 3.0 treatment, we speculate it to be in the vicinity of the Mn cluster, on the basis of our findings in this study that the low pH sensitive Ca atom regulates S-state transitions. In this context, it may be worthwhile to note our recent findings [36] that photoactivation in the presence of only Mn (but no Ca) induces miss ligation of Mn to a Ca-binding site, which results in formation of a Mn cluster incapable of S_2 to S_3 transition. Presumably, this Ca-binding site essential for functional photoactivation is equivalent to the low affinity Ca-binding site susceptible to pH 3.0 treatment revealed in this study to regulate the S-state turnovers.

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