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S-state turnover in the O_2-evolving system of $CaCl_2$-washed Photosystem II particles depleted of three peripheral proteins as measured by thermoluminescence. Removal of 33 kDa protein inhibits S_3 to S_4 transition

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The S-state transition in manganese-containing spinach PS II particles depleted of three peripheral proteins (33, 24 and 16 kDa) by $CaCl_2$ washing was investigated by means of thermoluminescence measurements and the following results were obtained: (1) When excited by continuous light, these particles showed the same glow curves as those of control PS II particles having a marked peak around +35°C (B band) which arises from recombination between S_2 (or S_3), the oxidized species of the so-called S states of the water-oxidation enzyme, and Q_B^-, the semiquinone form of the secondary plastoquinone acceptor of PS II. (2) When excited, however, by a series of flashes, oscillation of the B band in the depleted particles proceeded normally up to the 2nd flash but was interrupted thereafter; in contrast, the control particles underwent quadruple oscillation showing maxima at the 1st and 5th flashes. (3) The oscillation pattern of the depleted particles agreed well with a computer simulation pattern obtained by assuming inhibition of the $S_3 \rightarrow S_4$ transition. (4) The B-band height created by one or two flashes in the depleted particles showed decay kinetics almost the same as those in control particles. (5) During incubation in a low-salt medium, the B-band height of the depleted particles gradually decreased concomitant with release of manganese from the particles, and reached a zero level when about half of the manganese atoms were lost. (6) Removal of the 24 and 16 kDa proteins by NaCl washing appreciably lowered the B-band height, but did not affect at all the oscillation pattern of the B-band. These results indicate that the manganese catalyst in $CaCl_2$-washed PS II particles is unable to undergo the $S_3 \rightarrow S_4$ transition because of depletion of the 33 kDa protein, while the catalyst is still capable of undergoing $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ transitions.

Introduction

Three peripheral proteins with molecular masses of 33, 24 and 16 kDa are thought to be involved in O_2 evolution based on many disintegration and reconstitution experiments with O_2-evolving PS II particles in recent years [1–5]. Of these three proteins, the 24 and 16 kDa proteins are removed from the particles by washing with concentrated NaCl concomitant with partial inactivation of O_2 evolution, and the inactivation can be reversed either by rebinding of only the 24 kDa protein [2,3] or both the 24 and 16 kDa proteins [5]. Recently, a similar restoration was shown to occur on addition of Cl^- and/or Ca^{2+} to the particles depleted of the 24 and 16 kDa proteins [6–9]. It was suggested, therefore, that the two proteins afford high-affinity binding of Ca^{2+} and/or Cl^-

Abbreviations: PS, Photosystem; Mes, 4-morpholineethanesulfonic acid; Chl, chlorophyll; Z, the secondary donor of Photosystem II.

at the O_2-evolving center [7–9].

As to the role of 33 kDa protein, we reported that total liberation of the three proteins (33, 24 and 16 kDa) by washing with concentrated $CaCl_2$ results in complete inactivation of O_2 evolution even though all the manganese atoms are preserved in the particles [10], and O_2 evolution can be restored by rebinding of the 33 kDa protein to the washed particles [4]. We have also reported that in the absence of the 33 kDa protein, about half of the manganese atoms preserved in the washed particles are destabilized and gradually liberated during incubation in a low-salt medium, but the release can be suppressed by the presence of 50 mM $CaCl_2$ in the suspension medium concomitant with partial restoration of O_2 evolution [11]. Based on these findings, we proposed that the role of the 33 kDa protein is to maintain the conformation of the catalytic manganese atoms required for water oxidation [11].

In our previous studies, the functional activity of these proteins and manganese atoms was estimated primarily in terms of O_2 evolution, the overall process of the linear four-step mechanism of water oxidation [12,13]. Since the manganese atoms present in $CaCl_2$-washed particles are retained in a native binding environment and their catalytic function can be restored on rebinding of the 33 kDa protein [4], we expect that the manganese atoms undergo some of the partial reactions involved in the linear four-step model. In this study, we applied thermoluminescence measurements to detect such partial reactions. Thermoluminescence B-band peaking at 25–40°C arises from recombination of the electrons on the semiquinone form of the secondary plastoquinone acceptor, Q_B, with positive charges on the water-oxidation enzyme, S_2 and S_3 [14–17]. Thus, thermoluminescence measurements enable us to detect the charge pairs created as a result of single electron transfer from manganese to Q_A or Q_B independent of O_2 evolution. The results obtained indicate that the 33 kDa protein is required for the transition from S_3 state to S_4 state.

Materials and Methods

The O_2-evolving PS II particles were prepared from spinach as in Ref. 18, washed three times with 400 mM sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 6.5) and resuspended in 400 mM sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 5.5). In order to afford complete relaxation on both the donor and acceptor sides of PS II, the particles were incubated at 0°C in darkness for 5 h, and then the following treatments were carried out under dim green safe light.

$CaCl_2$ washing. The isolated particles were suspended in 1.2 M $CaCl_2$/400 mM sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 6.5) in darkness as described previously [10], and centrifuged at $35\,000 \times g$ for 10 min. The resulting pellet was washed once with 400 mM sucrose/40 mM Mes-NaOH (pH 5.5)/200 mM NaCl, and then resuspended in the same medium at a chlorophyll concentration of 5 mg Chl/ml. Inasmuch as liberation of the 33 kDa protein proceeds more slowly in complete darkness [19], the concentration of $CaCl_2$ was increased by 20% as compared with that in previous studies. The presence of 200 mM NaCl in the final suspension was effective in stabilizing the bound manganese atoms in the 33 kDa protein-depleted particles. The $CaCl_2$-washed particles thus obtained were diluted with 400 mM sucrose/40 mM Mes-NaOH (pH 5.5)/10 mM NaCl at a chlorophyll concentration of 0.25 mg Chl/ml just before the thermoluminescence measurements. A further 10 min dark incubation at 0°C preceded the excitation for thermoluminescence.

NaCl washing. The PS II particles were suspended in 1 M NaCl/400 mM sucrose/40 mM Mes-NaOH (pH 6.5) in darkness as described previously [10], and centrifuged at $35\,000 \times g$ for 10 min. The resulting pellet was washed once with 400 mM sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 5.5) and resuspended in the same medium at a chlorophyll concentration of 0.25 mg Chl/ml, and kept in darkness until thermoluminescence measurements.

Removal of Mn from $CaCl_2$-washed particles. The $CaCl_2$-washed particles were suspended in a low-salt medium containing 400 mM sucrose/10 mM NaCl/40 mM Mes-NaOH (pH 5.5) at a chlorophyll concentration of 0.25 mg Chl/ml and incubated at 0°C in darkness. After various period of incubation, an aliquot of this suspension was used for thermoluminescence measurements and manganese determination.

Thermoluminescence measurements. Samples (0.08 ml aliquot of the particle suspension at 0.25 mg Chl/ml) were illuminated either with continuous red light ($\geqq$ 630 nm) for 1 min at an intensity of about 0.7 mW/cm^2 at various low temperatures or with a series of flashes provided from a xenon strobe (5 μs, 4.5 J, white light) at 25°C at an interval of 1 s. The illuminated samples were quickly cooled to 77 K as described in Refs. 15 and 17. Computer simulation was done by the method described in Refs. 14 and 20 with a Hewlett Packard microcomputer model 85F.

Other measurements. O_2 evolution was measured with a Clark-type oxygen electrode with 2,5-dimethylquinone as electron acceptor at 25°C in 400 mM sucrose/10 mM NaCl/2 mM 2,5-dimethylquinone/40 mM Mes-NaOH (pH 6.5) as in Ref. 10 with supplement of salts when indicated. The abundance of manganese was determined with a Shimadzu atomic absorption spectromer (AA-640-13) as in Ref. 10.

Results

Fig. 1 shows the thermoluminescence glow curves of normal and $CaCl_2$-washed PS II particles excited by continuous illumination at three different temperatures. The control particles (Fig.

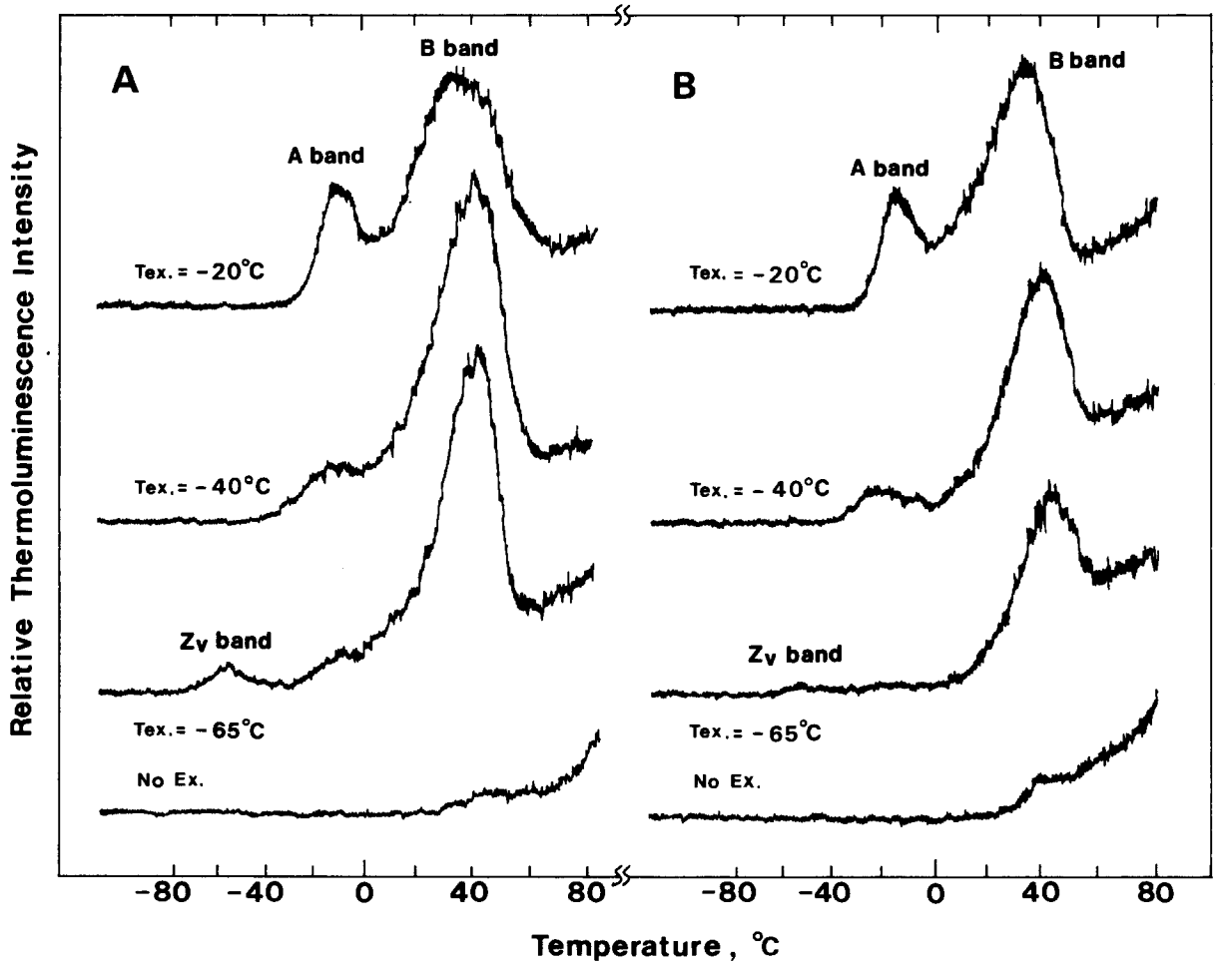

Fig. 1. Thermoluminescence glow curves of control (A) and 1.2 M $CaCl_2$-washed (B) PS II particles. Samples were illuminated with continuous red light for 1 min at three different temperatures of $T_{ex} = -20$, -40 and -65°C for upper, middle and bottom glow curves, respectively. O_2-evolving activities of the control and washed particles were 660 and 23 μmol O_2/mg Chl per h, respectively.

1A) excited at −65°C showed a strong B band around +35°C and weak Z_v and A bands at −55 and −10°C, respectively. When excited at −40°C, the Z_v band shifted to higher temperatures and partially overlapped with the A band. At a higher excitation temperature of −20°C, the Z_v band overlapped completely with the A band and the peak position of the B band shifted to a lower temperature concomitant with a broadening of the band. These features of the glow curves of PS II particles are similar to those of isolated chloroplasts measured at pH 7.4 (Ref. 17).

The glow curves of $CaCl_2$-washed particles measured under the same excitation conditions are shown in Fig. 1B. All the curves are in good agreement with respective glow curves of control particles, except that the B-band heights of washed particles are slightly lower than those of control particles (on a chrolophyll basis). According to the recent view about the origin of glow peaks, the B band is considered to be emitted as a result of charge recombination between S_2 and Q_B^- of S_3 and Q_B^- [15,16]. Thus, the similarity in glow curves between the two PS II particles indicates that PS II photochemistry occurs almost normally in $CaCl_2$-washed particles to yield charge pairs of $S_2Q_B^-$ and $S_3Q_B^-$, even though the particles have lost 97% of the O_2-evolving activity by removal of the three peripheral proteins.

The B band was observed to undergo quadruple oscillation [21], and the mechanism for the oscillatory behavior has been analyzed in more detail [15,20]. Fig. 2 shows the oscillatory pattern of the B band after a series of flashes in normal and $CaCl_2$-washed PS II particles. As seen by the pattern in Fig. 2A, the B-band height of control particles showed a marked dependence on flash number, with maxima at the 1st and 5th flashes and a minimum at the 4th flash. This pattern agrees with those previously observed for the B band [15] or the delayed luminescence component in several 10 s time scale [16] of well dark-adapted chloroplasts.

$CaCl_2$ washing affected the oscillation pattern in two ways. First, the B-band height after the 1st and 2nd flashes was lowered to 80% (on a chlorophyll basis) as compared with the height in control particles, and second, the marked oscillatory behavior after the 3rd flash was abolished to show a

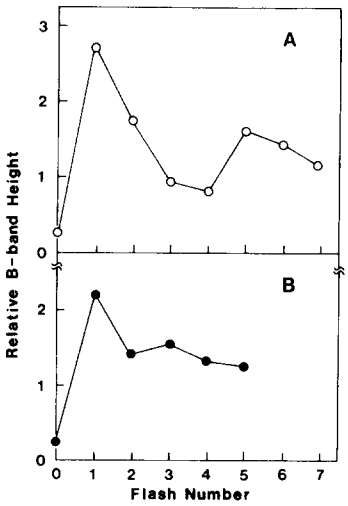

Fig. 2. Oscillation of flash-induced thermoluminescssence B band in control (A) and 1.2 M $CaCl_2$-washed (B) PS II particles. Dark-adapted samples were illuminated with a series of flashes at 25°C and cooled quickly to 77 K. O_2-evolving activities of the control and washed particles were 750 and 23 μmol O_2/mg Chl per h, respectively.

monotonously declining pattern with no second maximum at the 5th flash (Fig. 2B). These results indicate that $CaCl_2$-washed particles undergo normal S-state transition at flash numbers lower than 2, even though the O_2 evolution of the particles is almost completely inactivated by removal of the three peripheral proteins.

The above oscillation patterns are compared with the result of computer simulation. As shown in Fig. 3A, the oscillation pattern predicted for the control sample agreed well with the observed pattern. The following conditions were assumed in the simulations: 8% misses; 5% double hits; initial states of reaction centers, 12.5% S_0Q_B, 12.5% $S_0Q_B^-$, 62.5% S_1Q_B, 12.5% $S_1Q_B^-$ ($S_1 : S_0 = Q_B : Q_B^- = 75 : 25$); ratio of luminescence yield between $S_3Q_B^-$ and $S_2Q_B^-$ recombinations = 2.0. The details and relevance for these assumptions are published elsewhere [14,20]. In simulation for the washed particles, the presence of active centers (3%) was taken into account based on the inhibition (97%) of O_2 evolution caused by $CaCl_2$ washing.

In Fig. 3B, the oscillation pattern of $CaCl_2$-washed particles is compared with four predicted patterns (a–d) in which transitions of S states are

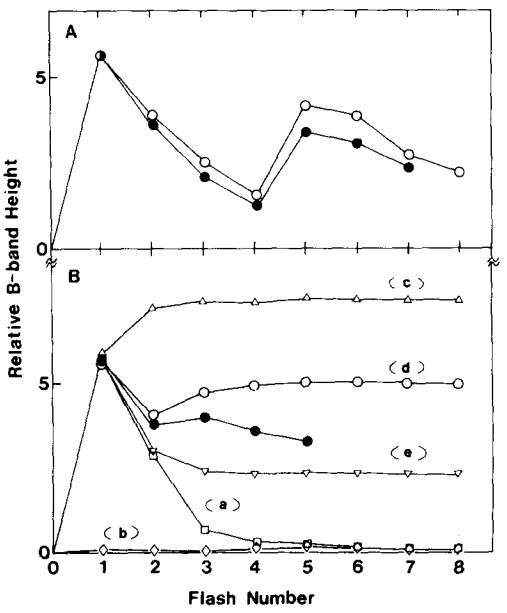

Fig. 3. Computer simulation of thermoluminescence B-band height oscillation. A, predicted (open circles) and observed (solid circles) patterns in control PS II particles. B, predicted patterns (open symbols) obtained by assuming inhibitions of $S_0 \rightarrow S_1$ (a), $S_1 \rightarrow S_2$ (b), $S_2 \rightarrow S_3$ (c), $S_3 \rightarrow S_4$ (d) transitions, and dual inhibition of $S_3 \rightarrow S_4$ plus $S_0 \rightarrow S_1$ (e) transitions. The pattern with solid circles is that observed for 1.2 M $CaCl_2$-washed PS II particles. Parameters used for simulation were 8% misses, 5% double hits, initial state of reaction centers, 12.5% S_0Q_B, 12.5% $S_0Q_B^-$, 62.5% S_1Q_B, 12.5% $S_1Q_B^-$ ($S_1:S_0 = Q_B:Q_B^- = 75:25$), and luminescence yield of $S_3Q_B^-/S_2Q_B^- = 2.0$. In simulation for the washed particles, 3% of the residual active centers were taken into account based on the inhibition extent of 97% (see Fig. 2 legend).

assumed to be inhibited at S_0, S_1, S_2 and S_3, respectively. Among the four predicted patterns, the pattern (d) simulated by assuming the inhibition of transition from S_3 to S_4 ($= S_0$) agreed with the observed pattern better than any other simulated patterns. This result suggests that removal of the three peripheral proteins by $CaCl_2$ washing results in inhibition of the $S_3 \rightarrow S_4$ transition.

A question may arise whether or not the inhibition is specific for the $S_3 \rightarrow S_4$ transition. Although the specific inhibition of the other transitions, $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$, could be ruled out since the respective predicted patterns differ so much from the observed pattern, there remains an ambiguity whether the inhibition of the S_3 transition is accompanied by the inhibition of the S_0 transition. The predicted pattern obtained by such an assumption is shown by pattern (e), which also considerably resembles the observed pattern. Judging, however, from the better fit with respect to the B-band height after the 2nd flash, it seems likely that the inhibition does not involve $S_0 \rightarrow S_1$ interruption. We may thus consider that the main cause for the interruption of oscillation after the 3rd flash is cuased solely by the inhibition of $S_3 \rightarrow S_4$ transition.

Fig. 4 shows the decay kinetics of $S_2Q_B^-$ (A) and $S_3Q_B^-$ (B) charge pairs created in normal and $CaCl_2$-washed particles. The B-band height after one flash ($S_2Q_B^-$) showed a monophasic decay with an approximate half-time of 90 s both in normal

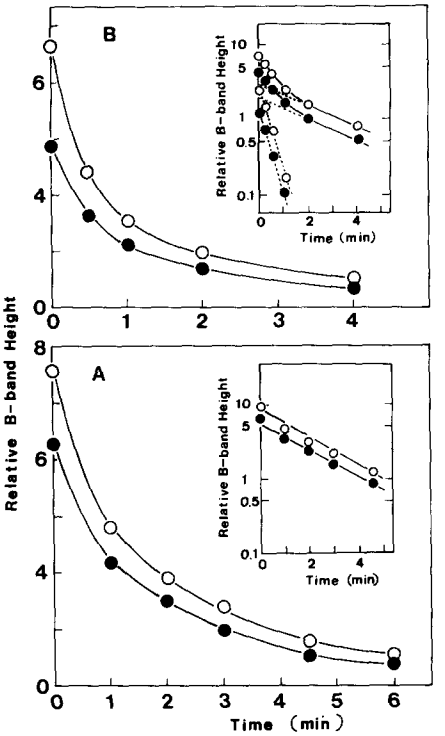

Fig. 4. Decay courses of the charge pairs of $S_2Q_B^-$ (A) and $S_3Q_B^-$ (B) in control (open circles) and 1.2 M $CaCl_2$-washed (solid circles) PS II particles. Samples were illuminated with one (A) or two (B) flashes and cooled to 77 K after varied period of the dark incubation at 25°C. The B-band height observed was plotted against the time of incubation. Insets are semilogarithmic plots. Note the different time scales for A and B. O_2-evolving activities of the control and washed particles were 660 and 23 μmol O_2/mg Chl per h, respectively.

and $CaCl_2$-washed particles (Fig. 4A), whereas the B-band height after two flashes ($S_3Q_B^-$) showed a biphasic decay with approximate half-times of 20 and 90 s both in normal and $CaCl_2$-washed particles (Fig. 4B). The similarity of the decay curves between normal and $CaCl_2$-washed particles indicates that the stability of the charge pairs of both $S_2Q_B^-$ and $S_3Q_B^-$ is not much affected by $CaCl_2$ washing. This implies that the three peripheral proteins are involved neither in creation by photoreaction nor in stabilization of S_2 and S_3 states.

Fig. 5 shows the relationship between manganese abundance and thermoluminescence intensity. As we have previously reported [11], half of the manganese atoms preserved in $CaCl_2$-washed particles are destabilized because of the absence of the 33 kDa protein. In order to confirm that thermoluminescence arises from the positive charges stabilized on manganese atoms, we investigated the flash-induced B-band height during the course of manganese release. As shown in Fig. 5A, when $CaCl_2$-washed particles were incubated in a low-salt medium, manganese abundance in the particles decreased gradually to approximately half of the original abundance after 12 h of incubation. When the B-band height after one or two flash illuminations was measured for the particles during the incubation, the curves in Fig. 5B were obtained. The B-band height decreases with a time course similar to the loss of manganese, indicating a close correlation between manganese abundance and thermoluminescence. In accordance with the oscillation pattern shown in Fig. 3, the B band after one flash was always higher than that after two flashes, and the ratio of the two B-band heights remained approximately constant at a value of 0.6 (Fig. 5B inset), which is close to the predicted ratio of 0.7 (Fig. 3). The constant ratio indicates that all the S_2 states created by the 1st flash can be converted by the 2nd flash to S_3 states, even if the number of centers capable of creating S_2 state decreases with release of manganese. This implies that the site of inhibition caused by release of manganese cannot be $S_2 \rightarrow S_3$ transition but $S_1 \rightarrow S_2$ transition; namely, manganese release causes complete loss of positive charge storing capability of the centers.

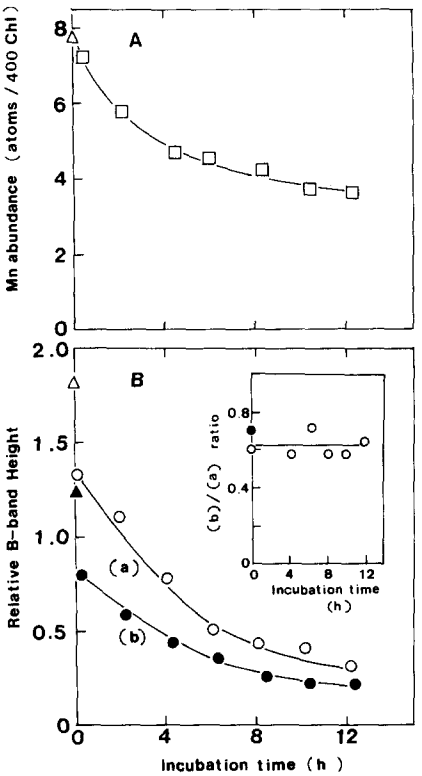

Fig. 5. Kinetics of decrease in manganese content (A) and thermoluminescence intensity (B) during incubation of 1.2 M $CaCl_2$-washed PS II particles in a low-salt medium at 0°C. The B-band height after one (a) or two (b) flashes and manganese content were plotted against the incubation time. Triangles in A and B are the data obtained with control PS II particles. Inset is the ratio of B-band height after two flashes relative to one flash. The solid circle in the inset is the theoretical ratio predicted by the data in Fig. 3. O_2-evolving activity of the washed particles was 22 μmol O_2/mg Chl per h and the activity before washing was 620 μmol O_2/mg Chl per h.

In Fig. 6, the relationship between manganese abundance and thermoluminescence intensity was further analyzed using the data in Fig. 5 experiment. Both B-band heights after one or two flashes plotted against manganese abundance showed straight lines with a commom extrapolated intersect at about 3 Mn/400 Chl. This implies that the thermoluminescence capability is completely lost when about half of the manganese atoms preserved in $CaCl_2$-washed particles are removed from the O_2-evolving center, which agrees with our previous view [11] that half of the four manganese atoms are directly involved in water oxidation.

The oscillation pattern of thermoluminescence in NaCl-washed PS II particles is shown in Fig. 7.

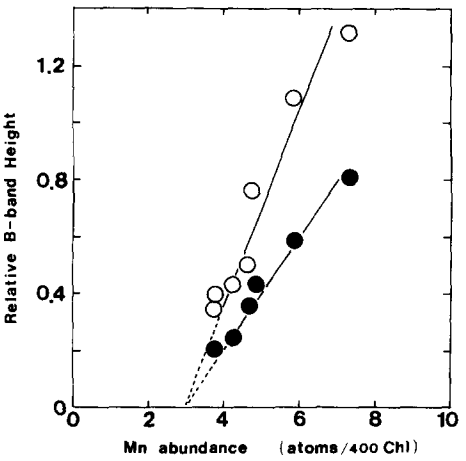

Fig. 6. Relationship between manganese content and thermoluminescence intensity. Open and solid circles are the B-band heights after one flash and two flashes, respectively.

By NaCl washing, two peripheral proteins (24 and 16 kDa) are removed while the 33 kDa protein and all manganese atoms remain associated with the particles, and the O_2-evolving activity is inactivated by about 50%. As shown by the two patterns in Fig. 7, the oscillation is not affected at all by NaCl washing and shows a quadruple pattern with maxima at the 1st and 5th flashes; however, there is a slight reduction in the B-band height. The extent of reduction estimated from the B-band height after one flash was 31% on a chlorophyll basis.

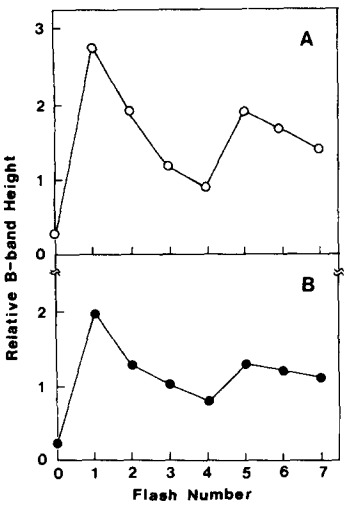

Fig. 7. Oscillation of flash-induced thermoluminescence B band in control (A) and 1.0 M NaCl-washed PS II particles (B). The procedures were the same as in Fig. 2. O_2-evolving activities of the control and washed particles were 690 and 350 μmol O_2/mg Chl per h, respectively.

From the fact that the washed sample undergoes normal oscillation showing the second maximum at the 5th flash, we consider that the inhibition effected by NaCl washing is of an all-or-none type. Since any inhibition of S-state transition would result in some distortion of the oscillation pattern if it is assumed to occur impartially in all centers, the only alternative interpretation may be that NaCl washing cuases a complete inhibition of $S_1 \rightarrow S_2$ transition in 30–40% of the centers, possibly by affecting the electron transport from manganese to Z, the secondary donor in the PS II photochemical reaction. In this case also, we have to assume an all-or-none type inhibition: if we assume that the quantum yield of the electron transport from manganese to Z is lowered to about half by NaCl washing, it implies a tremendous increase in misses factor, which will result in strong damping of the oscillation pattern. Since this is clearly not the case, the inhibition would be of an all-or-none type. Namely, the electron transport from manganese to Z is completely inhibited by NaCl washing in 30–40% of the centers, while in the other centers every reaction proceeds normally as in the control particles.

Discussion

The results presented demonstrate that in $CaCl_2$-washed PS II particles, which are incapable of O_2 evolution in spite of high manganese abundance (7–8 Mn/400 Chl) but depleted of the 33, 24 and 16 kDa proteins, the transition from S_3 to S_4 is specifically inhibited, although the transitions from S_0 to S_3 via S_1 and S_2 proceed normally (Figs. 2 and 3). We can thus attribute the loss of O_2 evolution of these particles to the interruption of the oxygen-clock at the S_3 state. The results suggest that the role of the 33 kDa protein is related to the conversion of S_3 to S_4.

The deactivation kinetics of both S_2 and S_3 states created in $CaCl_2$-washed particles were not much different when compared with those in un-

treated particles (Fig. 3). This implies that the stability of both S_2 and S_3 states is not affected by removal of the 33 kDa protein. This result is somehow unexpected. Since the functional manganese atoms are destabilized in the washed particles (Ref. 11, and this paper), some alteration in the properties of the S_3 state was expected as the cause for the inhibition of $S_3 \rightarrow S_4$ transition.

Considering these results along with our previous proposal about the role of the 33 kDa protein that the protein maintains the conformation of the functional manganese atoms as required for O_2 evolution [4,10,11], we can speculate that:

(1) The conformation of the manganese catalyst required for $S_3 \rightarrow S_4$ transition is not the same as required for $S_0 \rightarrow S_1 \rightarrow S_2 \rightarrow S_3$ transitions.

(2) The conformation required for $S_0 \rightarrow S_3$ transitions is maintained in the washed particles even in the absence of the 33 kDa protein.

(3) Depletion of the 33 kDa protein neither inhibits the formation nor affects the properties of S_2 and S_3 states, but strictly inhibits the transition from S_3 to S_4.

Thus, the role of the 33 kDa protein is not simply to stabilize as a whole the functional manganese atoms in the O_2-evolving center but is to maintain the conformation of the final intermediate species formed during $S_3 \rightarrow S_4$ conversion necessary for evolution of molecular O_2.

According to the model by Kusunoki [22], S_3 is the first S state in which an O-O bond is formed between the 2 oxygen atoms derived from the 2 water molecules present in an O_2-evolving center. Thus, the transition of S_3 state may differ from those of other S states in that it involves electron abstraction from hydrogen peroxide, while the others involve electron abstraction from water, and requires some special conformation of the manganese catalyst or the Mn-H_2O_2 complex. Although the detailed molecular mechanism awaits further investigations, we may assume that the 33 kDa protein provides the O_2-evolving center with this special conformation to stabilize the final intermediate species.

The present study also showed that thermoluminescence B band decreases when the manganese atoms release from the particles (Fig. 5), which appears to suggest that the positive charges for thermoluminescence B band are stabilized on manganese atoms. This view is consistent with previous observations that the B band disappears in manganese-deficient algal cells but reappears on addition of $MnCl_2$ under illumination [23]. In agreement with previous observations [11,24], the quantitative analysis (Fig. 6) suggests that about half of the manganese atoms preserved in the washed particles are involved in thermoluminescence and provide the catalytic function. Judging from the almost parallel courses of thermoluminescence decrease and manganese release, the 2 functional Mn atoms seem to be released from the particles as a pair or successively one after another. If the thermoluminescence capability is destroyed when the center loses one of the four manganese atoms, we should observe a thermoluminescence decrease faster than manganese release. The constant ratio between the B-band height after one flash and two flashes (Fig. 5B inset) appears to suggest paired manganese release.

The $CaCl_2$-washed PS II particles are depleted of the 24 and 16 kDa proteins in addition to the 33 kDa protein, so that the effect arising from depletion of the former two proteins must be taken into account in interpreting the present results. Wensink et al. [25] reported that removal of the 24 and 16 kDa proteins by NaCl washing causes an inhibition of $S_2 \rightarrow S_3$ transition. However, if the role of these two proteins is to provide the O_2-evolving center with a high-affinity binding site for Ca^{2+} and/or Cl^- as proposed by Ghanotakis et al. [7–9,26], they may have observed the effect of Cl^- depletion which is known to affect the transition of $S_2 \rightarrow S_3$ [26–28]. This interpretation is plausible because they measured X-320 (Q) absorption change in the absence of Cl^-, and the Cl^- demand would be much enhanced in the absence of the 24 and 16 kDa proteins [9].

Since a similar enhancement in Cl^- demand was expected in $CaCl_2$-washed particles, all the thermoluminescence measurements were made in the presence of 10 mM NaCl, which is sufficient to completely reverse the Cl^- depletion effect in the absence of the 24 and 16 kDa proteins [9]. According to Refs. 26–28, Cl^- depletion causes inhibition of $S_2 \rightarrow S_3$ transition accompanied by marked retardation of S_2 deactivation. However, owing to

TABLE I

PARTIAL RESTORATION BY Cl^- AND Cl^- PLUS Ca^{2+} OF O_2 EVOLUTION IN 1.2 M $CaCl_2$-WASHED PS II PARTICLES

	O_2 evolution (μmol O_2/mg Chl per h)	Mn abundance (atoms/400 Chl)
Control particles		
No addition	610 (100%)	7.7
1.2 M $CaCl_2$-washed particles		
No addition [a]	17 (3%)	7.3
+ 200 mM NaCl	69 (11%)	–
+ 200 mM NaCl and 10 mM $CaCl_2$	123 (20%)	–

[a] Basal reaction medium contained 10 mM NaCl in order to reverse the enhanced Cl^- depletion brought about by removal of the 24 and 16 kDa protein [9].

the presence of 10 mM NaCl, we observed neither inhibition of $S_2 \rightarrow S_3$ (Figs. 2 and 3) nor retardation of S_2 deactivation (Fig. 4). We may thus consider that the inhibition of $S_3 \rightarrow S_4$ transition presented in this paper is caused solely by the depletion of the 33 kDa protein.

Recently, Ca^{2+} was shown to reverse the partial inactivation of O_2 evolution resulting from removal of the 24 and 16 kDa proteins [6–8]. This is also considered to arise from the enhanced Ca^{2+} demand in the particles depleted of the two proteins. In order to avoid this Ca^{2+} effect, addition of Ca^{2+} in the medium for thermoluminescence measurements seemed appropriate. We did not, however, add Ca^{2+} in the measurements since $CaCl_2$ (50 mM) appreciably restores O_2 evolution in $CaCl_2$-washed particles [11] and makes it impossible to observe the effect brought about by depletion of the 33 kDa protein. In Table I, the effects of Cl^- and Ca^{2+} on O_2 evolution by $CaCl_2$-washed particles are summarized. By removal of all the three proteins (33, 24 and 16 kDa), O_2 evolution was inactivated to 3% of the original rate. On increasing NaCl concentration to 200 mM, O_2 evolution was restored to about 10%, and the restoration was doubled by supplement of a low concentration (20 mM) of $CaCl_2$. Thus, both Cl^- and Ca^{2+} are effective in partially restoring O_2 evolution in PS II particles depleted of the 33, 24 and 16 kDa proteins. Our previous observation that 50 mM $CaCl_2$ appreciably restores O_2 evolution [11] is consistent with this interpretation.

Three Cl^- effects may be distinguished: (i) classical Cl^- effect observed for chloroplasts [29], (ii) Cl^- effect observed for the 24 and 16 kDa protein-depleted PS II particles [9], (iii) Cl^- effect observed for the 33, 24 and 16 kDa protein-depleted PS II particles [24]. Effective concentrations for (i), (ii) and (iii) are 1–5 (Ref. 29), 5–10 (Ref. 9) and 200–300 mM (Ref. 24), respectively. As mentioned above, Cl^- effect (i) causes inhibition of $S_2 \rightarrow S_3$ transition [26–28]. According to the recent results by Nakatani [9] that 10 mM Cl^- reverses the partial inactivation of O_2 evolution caused by NaCl washing, (i) and (ii) are of the same origin, i.e., (ii) is an enhanced manifestation of (i) caused by removal of the 24 and 16 kDa proteins. As to (iii), Miyao and Murata [24] observed a partial restoration of O_2 evolution by 200 mM NaCl in urea-treated PS II particles and suggested that in the 33 kDa protein-depleted particles, the Cl^- demand is still more enhanced. This idea seems, however, unlikely in view of the present results. As we discussed above, removal of the 33 kDa protein inhibits O_2 evolution by interrupting the S state transition at S_3, so that the partial restoration of O_2 evolution by high concentration of Cl^- or by Cl^- plus Ca^{2+} (Refs. 11 and 24 and Table I) must have resulted from reversing the inhibition of $S_3 \rightarrow S_4$ transition, but not from reversing the inhibition of $S_2 \rightarrow S_3$. Thus Cl^- effect (iii) differs in mechanism from Cl^- effects (i) and (ii).

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