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Effects of removal and reconstitution of the extrinsic 33, 24 and 16 kDa proteins on flash oxygen yield in Photosystem II particles

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The effects of removal and reconstitution of the three extrinsic proteins on the flash O_2 yield were investigated and the following results were obtained. (1) Removal in darkness of the 24 and 16 kDa proteins affected neither the oscillation pattern nor the signal amplitude of the flash O_2 yield. However, the signal amplitude was reduced with a factor of 2 in the presence of EDTA and was restored by excess Ca^{2+}. The EDTA treatment did not change the oscillation pattern of the flash O_2 yield, but considerably damped the oscillation pattern of thermoluminescence B band. These results suggest a heterogeneity among the centers in binding affinity for Ca^{2+}, and that Ca^{2+} removal induces an all-or-none type inactivation of O_2 evolution but not in the thermoluminescence processes, indicative of an inhibition of the S-state turnover at a specific S-state. (2) Removal in darkness of the 33, 24 and 16 kDa proteins abolished the flash O_2 yield, but the inhibited yield was appreciably restored either by reconstitution with the 33 kDa protein or by inclusion of 200 mM Cl^- in the reaction mixture. The flash O_2 yield reconstituted by the 33 kDa protein exhibited a rather normal oscillation pattern accompanied by a slightly increased damping, which could be simulated by assuming a high miss factor (30%) for $S_3 \rightarrow S_0$ transition. The Cl^--restored flash O_2 yield exhibited a strongly damped oscillation pattern with obscured maxima at the 4th and 8th flashes, which was simulated by assuming a much higher miss factor (70%) for $S_3 \rightarrow S_0$ transition. It was indicated that the Cl^--restored O_2 evolution considerably differs from the 33 kDa protein-reconstituted O_2 evolution with respect to the mechanism of S-state turnover.

Introduction

The O_2-evolving system of higher plants includes three extrinsic proteins with approximate molecular masses of 33, 24 and 16 kDa [1–3]. Through recent technical development and improvement for specific removal and reconstitution of these proteins in isolated PS II particles [1–5], part of the functional roles of these extrinsic proteins have been revealed [1–21].

Removal of the 24 and 16 kDa proteins by NaCl-wash inactivates about half [6] or large part [12] of the O_2-evolving activity, and the inactivation can be restored by the presence of Ca^{2+} [7–9] as well as by reconstitution with the 24 kDa protein [2,6]. Based on the observation that activity reconstitution by rebinding of the 24 kDa protein is possible only in the presence of Ca^{2+}, Ghanotakis et al. [9] proposed a view that the role of the protein is to afford a high affinity binding site for Ca^{2+} in the O_2-evolving center, i.e., in the

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

presence of the protein, the functional Ca^{2+} is tightly bound to the particles, being protected from the attack by chelators, whereas in its absence, the Ca^{2+} binding affinity is lowered to allow removal of Ca^{2+} by the action of light [10] or chelators. In fact, the O_2 evolution by the PS II reaction center complex, which retains Mn and the 33 kDa protein but is depleted of the 24 and 16 kDa proteins, exhibits a strong demand for Ca^{2+} [11]. In contrast to this view, however, Boussac et al. [12] recently reported that a large part of the centers retain the high-affinity site for Ca^{2+} even after removal of the 24 kDa protein.

The site(s) of Ca^{2+} action in these reactivation experiments is not clear at present. Based on the characteristic effect of Ca^{2+} removal, an all-or-none type inhibition of the O_2-evolving activity [6,13], the action site has been considered to be located in the electron transfer around Z [10,14,15], the secondary donor of PS II. In contrast to these, recent results by Boussac et al. [16] showed that NaCl-washed particles are unable to go beyond S_3Z^+ state and Ca^{2+} restores the $S_3Z^+ \rightarrow S_0$ transition and O_2 evolution.

Removal of the 33, 24 and 16 kDa proteins by $CaCl_2$-wash totally inactivates O_2 evolution, even though all the Mn in the particles are preserved in the O_2-evolving centers [17]. Half of the Mn in the $CaCl_2$-washed particles is released during incubation in a low-salt medium [14,18], but the release is completely suppressed by rebinding of the 33 kDa protein concomitant with restoration of O_2 evolution [5,19,20]. This indicates that one of the functions of the 33 kDa protein is to maintain the Mn in the particles [14,18,21]. Thermoluminescence experiments with the $CaCl_2$-washed particles revealed that the washed particles are able to undergo normal S-state transitions to the S_3 state, but unable to undergo $S_3 \rightarrow S_0$ transition to evolve O_2 [14]. It was thus inferred that the 33 kDa protein also provides the Mn with a conformation specifically required for the $S_3 \rightarrow S_0$ transition. Similar suppression of Mn release is possible by inclusion of 50 mM $CaCl_2$ [18] or 200 mM NaCl [21] in the incubation medium. This indicates that a part of the function of the 33 kDa protein can be replaced by Cl^- [21]. In fact, an appreciable restoration of O_2 evolution has been shown to occur in the presence of high concentration of Cl^- [14,21]. However, the roles of Cl^- and Ca^{2+} for this O_2 evolution are totally unknown.

In the first part of this communication, we report the results of parallel measurement of the flash O_2 yield and thermoluminescence B band of NaCl-washed particles, and in the second part we compare the flash O_2 yield pattern of the two types of O_2 evolutions in $CaCl_2$-washed particles, one restored by rebinding of the 33 kDa protein and the other restored by the presence of high concentration of Cl^-.

Materials and Methods

Sample preparation. O_2-evolving PS II particles were prepared from spinach as described previously [14] according to the method by Berthold et al. [22], suspended in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) after three washes with the same medium, and then, incubated in darkness at 0°C for 12 h to afford complete relaxation on both the donor and acceptor sides of PS II. The relaxed particles were subjected to NaCl-wash or $CaCl_2$-wash under very dim green safe light.

NaCl-wash (30 min) was done in 1.5 M NaCl/400 mM sucrose/40 mM Mes-NaOH (pH 6.5) and the washed particles were resuspended in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) after two washes with the same medium. $CaCl_2$-wash (30 min) was done in 1.5 M $CaCl_2$/400 mM sucrose/40 mM Mes-NaOH (pH 6.5), and the washed particles were resuspended in 400 mM sucrose/200 mM NaCl/40 mM Mes-NaOH (pH 6.5) after two washes with the same medium. The 33 kDa protein was extracted from the NaCl-washed particles with 0.8 M Tris-NaOH (pH 9.3), concentrated (at pH 6.5) by ultrafiltration with an Amicon PM 10 Diaflo membrane, and then readded to the $CaCl_2$-washed PS II particles in 400 mM sucrose/200 mM NaCl/40 mM Mes-NaOH (pH 6.5). After 30 min dark incubation at 0°C, the reconstituted particles were pelleted, washed twice, suspended in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5).

Flash O_2 yield. The dark-adapted samples (0.25 mg Chl/ml) suspended in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) were

applied to a Joliot-type O_2-rate electrode [23] in complete darkness using an NEC noctovision goggle (NVR 2015), incubated for 0.5 min on the electrode, and then illuminated with a series of Xe flashes (4 μs; 2J) at a uniform interval of 1 s. The amperometric signal measured through the flow medium (20 mM NaCl) was amplified and the electronically differentiated signal was recorded. Computer simulation was done with a Hewlett Packard microcomputer (HP-9826) by assuming a fixed S_1/S_0 ratio of 0.75/0.25 with varying probabilities for misses and double hits.

Thermoluminescence measurements. The dark adapted samples (0.25 mg Chl/ml) suspended in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) were illuminated with a series of 5 μs Xe flashes at 25°C at an interval of 1 s, cooled rapidly to 77 K, and the glow curves were recorded as described in Ref. 14. Computer simulation was done with a Hewlett Packard microcomputer (HP-85F) as described previously [14].

Other measurements. O_2 evolution under continuous light was measured with a Clark-type oxygen electrode at 25°C in 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) as described in Ref. 17. The electron acceptor was either 2 mM 2,5-dimethyl-*p*-benzoquinone, 0.6 mM phenyl-*p*-benzoquinone or 0.6 mM 2,5-dichloro-*p*-benzoquinone. Protein composition was analyzed by SDS-polyacrylamide gel electrophoresis as described previously [17]. The relative amount of the extrinsic proteins was estimated by measuring the stained peak area on SDS-polyacrylamide gel electrophoresis densitograms.

Results

Effect of depletion of the extrinsic 24 and 16 kDa proteins

Fig. 1 shows the oscillation pattern of the flash O_2 yield of the PS II particles used in the present study. In the presence of ferricyanide as electron acceptor, native PS II particles showed a period-4 oscillation with a steady-state level of about 40% of the maximum yield at the 3rd flash. This pattern agrees with the typical oscillation reported for thylakoids [24], and can be fitted for the Joliot–Kok model by assuming a dark S-state distribution of 25% S_0 and 75% S_1, and 8% misses

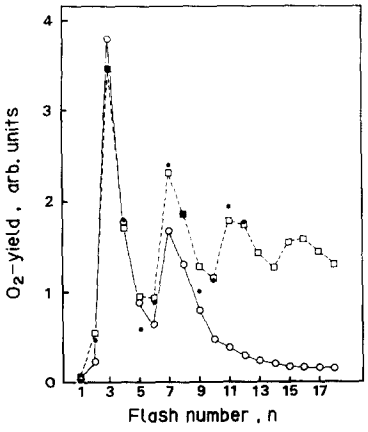

Fig. 1. Flash O_2 yield pattern of native PS II particles in the presence (□) and in the absence (○) of 0.5 mM ferricyanide. Predicted pattern (●) was obtained by assuming an initial S-state distribution of 75% S_1 and 25% S_0, and 8% misses and 5% double hits. Basal reaction medium contained 20 mM NaCl.

and 5% double hits for each turnover. Ferricyanide did not much affect the initial S_0/S_1 ratio, as opposed to the early report for thylakoids [25]. In the absence of ferricyanide, the oscillation proceeded normally during the first cycle but was damped from the second cycle on, and the signal amplitude quickly decreased with the increase in flash number. These features are essentially the same as those reported by Seibert and Lavorel [26] excepting a much steeper decrease of the yield towards the base line in our sample, indicative of a smaller size of the plastoquinone pool.

Fig. 2 shows the effect of 1.5 M NaCl-wash on flash O_2 yield pattern (in the absence of ferricyanide). SDS-polyacrylamide gel electrophoresis analysis confirmed that more than 90% of the 24 and 16 kDa proteins were removed in the washed particles (data not shown). Unexpectedly, the washed particles showed almost the same oscillation pattern with the same signal amplitudes (on Chl basis) as that of the control PS II particles (Fig. 2A and 2B). However, when 5 mM EDTA was included in the reaction medium, the signal amplitude was decreased to about half. Notably, this decrease in signal amplitude did not involve any change in the oscillation pattern (Fig. 2B and 2C). The signal amplitude thus decreased by 5 mM EDTA was restored by a further addition of

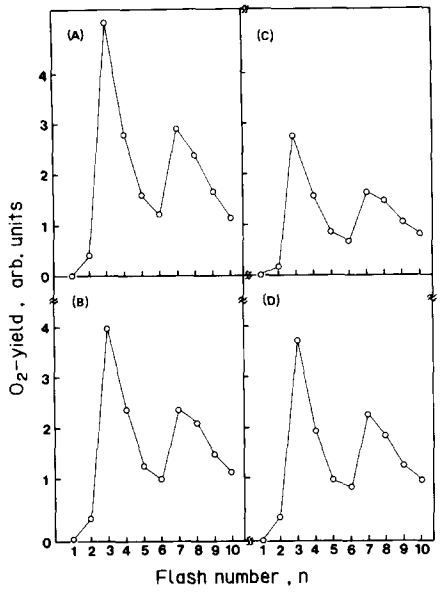

Fig. 2. Effect of NaCl-wash on the flash O_2 yield pattern by PS II particles. (A) Control particles (without ferricyanide); (B) 1.5 M NaCl-washed particles; (C) 1.5 M NaCl-washed particles plus 5 mM EDTA; (D) 1.5 M NaCl-washed particles with 5 mM EDTA plus 20 mM $CaCl_2$ after 5 min incubation. Basal reaction medium contained 20 mM NaCl.

TABLE I

EFFECTS OF EDTA AND $CaCl_2$ ON THE O_2 EVOLUTION BY 1.5 M NaCl-WASHED PS II PARTICLES MEASURED UNDER CONTINUOUS AND FLASH ILLUMINATIONS

For the continuous-illumination figures, the numbers between parentheses represent μmoles O_2 per mg Chl per h. The electron acceptor was 2 mM 2,5-dimethyl-*p*-benzoquinone. Light (of wavelength 420 nm or longer) intensity was saturating. For the flash illumination, the signal amplitudes of the flash O_2 yield (Fig. 2) during the first six flashes were integrated.

Sample	Addition	O_2 evolution (%)	
		continuous illumination	flash illumination
Control	None [a]	100 (790)	100
NaCl-washed	None	51 (400)	96
NaCl-washed	5 mM EDTA	51 (400)	54
NaCl-washed	5 mM EDTA and 20 mM $CaCl_2$ [b]	72 (570)	80

[a] Basal reaction medium contained 20 mM NaCl.
[b] $CaCl_2$ was added 5 min after EDTA addition.

20 mM $CaCl_2$ without changing the oscillation pattern (Fig. 2C and 2D). These results clearly indicate that the amplitude changes of the flash O_2 yield are due to Ca^{2+} effect, but not to Cl^- effect, since the enhanced Cl^- demand for O_2 evolution after NaCl-wash has been compensated by 20 mM NaCl in the reaction mixture [27].

Table I compares the effect of 1.5 M NaCl-wash on the rate of O_2 evolution under continuous and flash illuminations. Under continuous illumination the salt-wash exhibited an inhibition by about 50% and the inhibited activity was insensitive to 5 mM EDTA, but the inhibited activity was restored to 72% by the addition of 20 mM $CaCl_2$. The activity under flash illumination was estimated by integrating the flash O_2 yield shown in Fig. 2 during the first six flashes. The extents of activity inhibition by EDTA and restoration by $CaCl_2$ under flash illumination coincided well with those measured under continuous illumination. However, the activity under flash illumination after NaCl-wash without addition was much higher than that under continuous light. This indicates that EDTA is required to realize the salt-wash induced inhibition under flash illumination, but is not under continuous light.

From the flash O_2 yield pattern shown in Fig. 2, we can follow up the S-state turnover in those centers remaining active after NaCl wash. However, the flash O_2 yield measurement provides no information about the S-state transitions in the inactivated centers which do not evolve O_2. Thermoluminescence B-band has been assigned to arise from recombination of the electrons on the semiquinone form of the secondary plastoquinone acceptor, Q_B^-, with the positive charges on the S_2 and S_3 states [28,29], and its oscillation patterns have been analyzed in detail [28–30]. Thus, thermoluminescence measurements enable us to follow up the S-state turnover in the inhibited centers as well.

Fig. 3 shows the effect of 1.5 M NaCl wash on the oscillation pattern of flash-induced thermoluminescence B band. The B-band height of the control particles exhibited a marked dependence on flash number, showing maxima at the 1st and 5th flashes and a minimum at the 4th flash (Fig. 3A) as reported previously [14]. This pattern agrees with the oscillation pattern predicted by computer

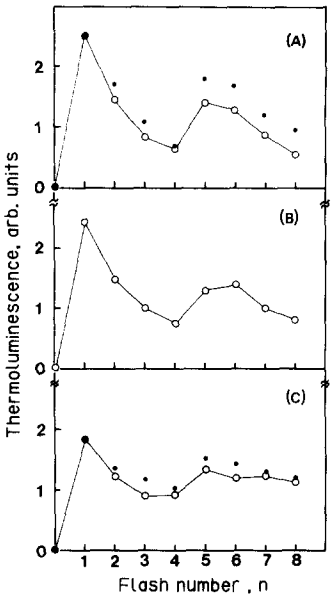

Fig. 3. Oscillation of flash-induced thermoluminescence B-band. (A) The B-band height of control particles (○) compared to the predicted pattern (●) obtained by assuming the initial state of reaction centers, 12.5% S_0Q_B, 12.5% $S_0Q_B^-$, 62.5% S_1Q_B, 12.5% $S_1Q_B^-$ ($S_1/S_0 = Q_B/Q_B^- = 75:25$), and 8% misses and 5% double hits, and luminescence yield of $S_3Q_B^-/S_2Q_B^- = 2.0$; (B) 1.5 M NaCl-washed particles; (C) 1.5 M NaCl-washed particles plus 5 mM EDTA (○) compared to the predicted pattern (●) obtained by assuming that 25% centers are totally dead, 25% centers are thermoluminescent, but are incapable of $S_3 \rightarrow S_0$ transition and 50% centers are totally healthy. Both washed and unwashed samples were suspended in a basal medium contained 20 mM NaCl.

simulation by assuming the following conditions: 8% misses, 5% double hits; initial redox 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 is 2.0 : 1.

The NaCl-washed particles showed an oscillation quite similar to the control particles with respect to both pattern and amplitude as shown in Fig. 3B. This is consistent with the results in Fig. 2 that NaCl wash itself affected neither the oscillation pattern nor the signal amplitude of the flash O_2 yield. The presence of 5 mM EDTA, however, affected the oscillation pattern as shown in Fig. 3C. The B-band height after the first flash was lowered, and the pattern was markedly damped and distorted, although some oscillatory behavior remained. This pattern contrasts to the normal oscillation of flash O_2 yield observed under the same condition (Fig. 2C), in which only the signal amplitude was decreased without changing the oscillation pattern. The different response between the flash O_2 yield and thermoluminescence B band can be satisfactorily accounted for, if we assume that some of the inactive centers incapable of O_2 evolution are still capable of undergoing some steps of S-state transitions to emit thermoluminescence (see Discussion).

Effect of depletion of the extrinsic 16, 24 and 33 kDa proteins

It is established that 1 M $CaCl_2$-wash completely removes the 16, 24 and 33 kDa proteins concomitant with total inactivation of O_2 evolution, and the 33 kDa protein can rebind to the washed centers to reconstitute O_2 evolution [5,19,20]. The extent of activity reconstitution by rebinding of the 33 kDa protein in our previous paper [5] was about 30% with dimethylbenzoquinone as acceptor. It turned out, however, that the extent of reactivation depends much on the species of the quinone acceptors used. As shown in Table II, the activity (at pH 6.5) of the control PS II particles depends on the species of acceptor quinones, showing the order, dimethyl-, phenyl-, dichlorobenzoquinone, whereas in reconstituted particles the activity was highest with dichlorobenzoquinone and decreased in the order, phenyl- and dimethylbenzoquinone. Thus the apparent extent of reactivation measured with dimethylbenzoquinone as acceptor was as high as 31–47%, but the extent amounted to 100% when measured with dichlorobenzoquinone (in the presence of 10 mM $CaCl_2$). The difference in the acceptor dependency before and after $CaCl_2$-wash suggests that $CaCl_2$-wash additionally affects the acceptor side of PS II and modifies the accessibility of quinone derivatives.

Fig. 4 shows the flash O_2 yield patterns before and after the $CaCl_2$-wash and reconstitution treatments. When 1.5 M $CaCl_2$-washed particles were measured in a low salt medium (20 mM NaCl), an anomalous pattern with a constant signal amplitude from the first flash was observed (B, open circles). When catalase (200 μg/ml) was included, the signal amplitudes were uniformly lowered and

TABLE II

ACCEPTOR DEPENDENCE OF THE RECONSTITUTION OF O_2 EVOLUTION IN 1.5 M $CaCl_2$-WASHED PS II PARTICLES BY REBINDING OF THE EXTRINSIC 33 kDa PROTEIN

O_2 evolution was measured in a basal medium containing 20 mM NaCl. The numbers between parentheses represent μmoles O_2 per mg Chl per h. As for continuous illumination, light (wavelength 420 nm or longer) intensity was saturating. Acceptor concentrations were 2 mM 2,5-dimethyl-*p*-benzoquinone, 0.6 mM phenyl-*p*-benzoquinone and 0.6 mM 2,5-dichloro-*p*-benzoquinone. As for flash illumination, signal amplitudes of the flash O_2 yield (Fig. 4) during the first six flashes were integrated.

Sample	O_2 evolution (%)			
	continuous illumination			flash illumination
	dimethyl benzoquinone	phenyl-benzoquinone	dichloro-benzoquinone	no artificial acceptor
Control	100 (858)	100 (728)	100 (681)	100
$CaCl_2$-washed	8 (47)	6 (47)	9 (60)	9
$CaCl_2$-washed 33 kDa-reconstituted	31 (270)	45 (329)	55 (376)	36
$CaCl_2$-washed 33 kDa-reconstituted (+10 mM $CaCl_2$) [a]	47 (399)	70 (511)	100 (681)	62

[a] Indicated salt was included in the reaction medium for O_2 evolution.

the anomalous signals at the first and second flashes were eliminated (B, open squares). Such effect of catalase has been reported by Åkerlund for NaCl-washed inside-out vesicles [31]. When the washed particles were reconstituted with the 33 kDa protein, the anomalous signals were eliminated without addition of catalase, and an oscillation pattern with maxima at the third and seventh flashes was appreciably restored (C). The restored signal amplitude was further enhanced by inclusion of 10 mM $CaCl_2$ (D) with no changes in the oscillation pattern. The extent of activity re-

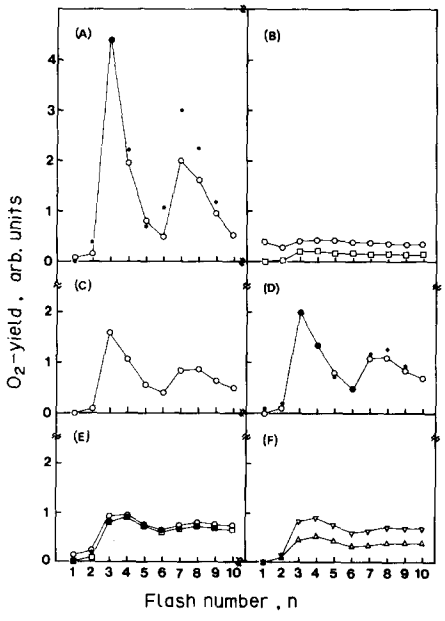

Fig. 4. Effect of $CaCl_2$-wash on the flash O_2 yield pattern by PS II particles. (A) Control particles (○) compared to the predicted pattern (●) obtained by assuming an initial S-state distribution of 75% S_1 and 25% S_0, and 8% misses and 5% double hits; (B) 1.5 M $CaCl_2$-washed particles, in the presence (□) and absence (○) of catalase (0.2 mg/ml); (C) 33 kDa protein-reconstituted particles in the absence of $CaCl_2$; (D) 33 kDa protein-reconstituted particles in the presence of 10 mM $CaCl_2$ (○) compared to the predicted pattern (●) obtained by assuming an initial S-state distribution of 75% S_1 and 25% S_0, and 30% misses for $S_3 \rightarrow S_0$ transition, and 8% misses and 5% double hits for the other transitions; (E) Cl^--reactivated particles (final concentration, 200 mM NaCl) in the presence (□) and absence (○) of catalase (0.2 mg/ml) compared to the predicted pattern (●) obtained by assuming an initial S-state distribution of 75% S_1 and 25% S_0, and 70% misses and no double hits for $S_3 \rightarrow S_4$ transition, and 8% misses and 5% double hits for the other transitions; (F) Cl^--reactivated particles (final concentration, 200 mM NaCl, and 0.2 mg/ml catalase) in the presence of 5 mM EDTA (△) or 5 mM EDTA plus 20 mM $CaCl_2$ (▽). Basal reaction medium contained 20 mM NaCl.

constitution estimated by integrating the signal amplitudes during the first six flashes was 36% and 62% in the absence and presence of 10 mM $CaCl_2$, respectively (Table II). These extents of reactivation measured under flashes (with no acceptor) are not as high as those measured under continuous illumination with dichlorobenzoquinone as acceptor, but are close to the values obtained with dimethylbenzoquinone or phenylbenzoquinone as acceptor. It seems that the activity recovery under flash illumination reflects realer reconstitution extents more specific to the donor side, since no artificial acceptors are added in flash O_2 yield measurements.

In spite of the peak positions at normal flash numbers (third and seventh), the flash O_2-yield pattern of the reconstituted particles was considerably damped. The pattern (in the presence of 10 mM $CaCl_2$) could be simulated by assuming the following conditions: dark S-state distribution of 25% S_0 and 75% S_1; 30% misses and 2% double hits for $S_3 \rightarrow S_4$ transition; 8% misses and 5% double hits for the other S-state transitions (D, solid symbols). More or less the same simulation is possible by assuming a uniform misses (15%) and double hits (3%) for each transition. In either case, an increased miss factor was necessary.

$CaCl_2$-washed particles lose half of their Mn during incubation in a low-salt medium [18,21]. However, if the incubation medium contains 50 mM $CaCl_2$ [18] or 200 mM NaCl [21], the release of Mn is suppressed and the particles show appreciable O_2 evolution [14,18,20,21]. The suppression of Mn release is chiefly due to high concentration of Cl^- [21] and co-existence of Ca^{2+} enhances the Cl^--reactivated O_2 evolution [14,20,21]. Fig. 4E and F show the flash O_2-yield pattern of the washed particles in the presence of 200 mM NaCl. In the absence of catalase, 200 mM NaCl induced an appreciable O_2 signal (E, open circles) superimposed on the anomalous signal as shown in (B). It is of note that 200 mM NaCl partially suppressed the anomalous signal, as seen from the lower signal amplitude at the first flash (compare the open circles in E and B at the first flash). When measured in the presence of catalase, the anomalous signal was eliminated (E, open squares) and an oscillatory pattern with appreciable signal amplitude was obtained. However, the oscillation pattern was much distorted, showing obscured maxima at the fourth and eighth flashes with a strong damping toward a relatively high steady-state level. The extent of activity restoration estimated by integrating the flash O_2 yield amounted to 37% (Table II), which is more than half of the reconstitution extent by the 33 kDa protein (62%). However, the oscillation pattern is very different from that of the 33 kDa protein-reconstituted particles, and the simulation of this pattern required a much higher miss factor: 70% misses with no double hit for $S_3 \rightarrow S_4$ transition, and 8% misses and 5% double hits for the other transitions. Although other simulations are possible, all of those for good fitting involve very high miss factors. These results clearly indicate that the S-state turnovers in the Cl^--reactivated O_2 evolution are considerably different from those in the 33 kDa protein-reconstituted O_2 evolution, in spite of the roughly comparable extent of activity restoration by the two reactivation methods.

Fig. 4F shows the effect of EDTA on the flash O_2 yield reactivated by 200 mM NaCl. When 5 mM EDTA was included in the reaction mixture, the signal amplitude was reduced to about 60% of the control without changing the oscillation pattern. When an excess amount of $CaCl_2$ (20 mM)

TABLE III

REACTIVATION OF O_2 EVOLUTION BY Cl^- IN $CaCl_2$-WASHED PS II PARTICLES

For the continuous illumination figures, the numbers between parentheses represent μmoles O_2 per mg Chl per h. The electron acceptor was 0.6 mM 2,5-dichloro-*p*-benzoquinone. Light (wavelength 420 nm or longer) intensity was saturating. For the flash illumination, the signal amplitudes of the flash O_2 yield (Fig. 4) during the first six flashes were integrated.

Sample	Addition	O_2 evolution (%)	
		continuous illumination	flash illumination
Control	None [a]	100 (666)	100
$CaCl_2$-washed	None	6 (38)	9
$CaCl_2$-washed	180 mM NaCl	33 (217)	38
$CaCl_2$-washed	180 mM NaCl + 10 mM $CaCl_2$	47 (311)	37
$CaCl_2$-washed	180 mM NaCl + 5 mM EDTA	31 (205)	20

[a] Basal reaction medium contained 20 mM NaCl.

was further included, the signal amplitudes recovered the initial level without changing the oscillation pattern, but the recovery did not go over the initial level observed without $CaCl_2$ addition (E). These effects of EDTA and $CaCl_2$ observed under flash illumination were somehow different from those observed under continuous illumination. As compared in Table III, addition of 10 mM $CaCl_2$ stimulated the O_2 evolution supported by 200 mM NaCl under continuous illumination, but did not under flash illumination. On the contrary, addition of 5 mM EDTA did not affect the O_2 evolution under continuous illumination while partially inhibited the O_2 yield under flash illumination. These results suggest that even after removal of all the three extrinsic proteins, the particles still retain a certain amount of functional Ca^{2+} which is removed by the action of continuous illumination or by the presence of EDTA.

Discussion

The present study demonstrated that the direct cause for the inactivation of O_2 evolution by NaCl-wash is not the removal of 24 and 16 kDa proteins but is the release of Ca^{2+} (Table I and Fig. 2). These results are largely consistent with the view by Ghanotakis et al. [7,9] that the 24 kDa protein affords a high-affinity site for Ca^{2+} in the O_2-evolving center, and are more closely consistent with the report by Boussac et al. [12] that the NaCl-washed particles mostly retain the high-affinity site for Ca^{2+} even in the absence of the 24 kDa protein, even though a precise comparison reveals some differences; EDTA was necessary to remove Ca^{2+} in our case, but not in theirs.

The difference may be due to the different light conditions during NaCl wash; in darkness in our case but in room light in theirs. As has been suggested by Dekker et al. [10], Ca^{2+} release from NaCl-washed particles is triggered by exposure to light, so that most of the 24 kDa protein depleted centers had lost Ca^{2+} in their case, but not in our case. The stable retention of Ca^{2+} during a dozen of flashes is consistent with the comment in [12] that a dark time (10 min) is required for a preflash to manifest its effect on flash O_2 yield in the residual O_2 evolution by the washed particles (in the absence of Ca^{2+}). Thus, the 24 kDa protein seems to afford the high affinity binding site for Ca^{2+} by protecting the site from detrimental effects by light.

In view of the observation that the inhibition extent (50%) under continuous illumination without EDTA coincides with the inhibition extent of the flash O_2 yield with EDTA (Table I), the light-triggered release of Ca^{2+} seems to take place only in half of the centers. This suggests either a heterogeneity in Ca^{2+} demand or a heterogeneity in Ca^{2+} affinity among the centers. From the various extents of inhibition by NaCl wash reported by various investigators [6,7,12], and from the fact that most part of those inhibitions are restored by Ca^{2+}, the latter explanation seems more likely. The residual O_2 evolution after NaCl wash in our experiment (50%) is notably higher than those by other investigators [12], but agrees with those by Miyao and Murata [6]. The reason for this discrepancy is not clear at present, but is possibly due to the differences in sample spinach and/or conditions for NaCl wash.

Removal of the functional Ca^{2+} from a center results in all-or-none type inhibition of O_2 evolution by the center. This is clearly concluded from the fact that both inactivation by EDTA and reactivation by excess Ca^{2+} did not induce any change in the oscillation pattern of the flash O_2 yield (Fig. 2). In contrast to these clear all-or-none type response of the flash O_2 yield, thermoluminescence oscillation was affected in a different way (Fig. 3): by the same EDTA treatment, the oscillation pattern of thermoluminescence was much more damped than that of the flash O_2 yield. The smaller degree of inhibition of thermoluminescence after the first flash implies that part of the Ca^{2+}-depleted centers is still able to undergo charge separation to emit thermoluminescence, although it is unable to undergo $S_3 \rightarrow (S_4) \rightarrow S_0$ transition to evolve O_2. This view is in good agreement with the recent results by Boussac et al. [16] that NaCl-washed centers cannot go beyond the S_3Z^+ state. Indeed, the computer simulation suggests that 25% of the centers cannot emit thermoluminescence (totally dead), 25% of the centers are able to emit thermoluminescence but are unable to go beyond the S_3 state to evolve O_2, and the other 50% of the centers are capable of

undergoing normal S-state transitions including O_2 evolution (totally healthy).

These analyses lead to a view that Ca^{2+} removal induces an all-or-none type inhibition in two different ways: one by disconnecting the S system from the reaction center, and the other by blocking the S-state transitions. The former site may be the electron transfer from Z to P-680$^+$ [10,15], and the latter site may be the transition from S_3 to S_0 as suggested by Boussac et al. [16]. The latter site may be related to the report by Ghanotakis et al. [32] that replacement of Ca^{2+} by lanthanoid induces some changes in Mn-binding conditions.

As opposed to NaCl wash, $CaCl_2$ wash in darkness resulted in almost complete inactivation of O_2 evolution. The inactivated O_2 evolution was appreciably restored either by reconstitution with the 33 kDa protein [5,19,20] or by inclusion of a high concentration of Cl^- [14,20,21] in the reaction mixture. When measured under continuous illumination, the extent of activity reconstitution by 33 kDa protein depended much on the species of electron acceptors, indicative of a modification on the acceptor side by $CaCl_2$ wash. Because of this effect, an almost 100% reactivation (in the presence of 10 mM $CaCl_2$) was possible with dichlorobenzoquinone as acceptor (Table II).

In contrast to such high extents of activity restoration observed under continuous light, the recovery of flash O_2 yield by reconstitution with 33 kDa protein was only 36% (measured with no Ca^{2+} and no artificial acceptor) in spite of 100% rebinding of the 33 kDa protein (Table II). However, the flash O_2 yield for this activity showed a marked oscillation, indicative of a satisfactory recovery of the S-state turnovers by reconstitution with 33 kDa protein (Fig. 4C). This suggests that a large part of the reconstituted centers is still incapable of O_2 evolution, probably because that part of the centers are devoid of a factor which effects the all-or-none type activation of the S-state turnover. This is clearly the case, since the flash O_2 yield was increased to 62% by the addition of 10 mM $CaCl_2$, notably without changing the oscillation pattern (Fig. 4C and D). These results indicate that successful reconstitution of the S-state turnover took place in 62% of the centers, and that the Ca^{2+} site was still retained in those centers and exhibited Ca^{2+} sensitivity. The other centers (38%) did not restore O_2 evolution even after reconstitution with the 33 kDa protein, probably due to the loss of Mn or a damage in the Ca^{2+} site during reconstitution procedures.

The oscillation pattern of the flash O_2 yield by the 33 kDa protein-reconstituted particles was respectably similar to the normal pattern, but the simulation required an enhanced miss factor for the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. This appears consistent with our previous report that removal of the 33 kDa protein induces a specific inhibition of the $S_3 \rightarrow S_4$ transition, and the enhanced miss factor may imply that the rebinding of the 33 kDa protein does not always effect a complete restoration of the inhibited transition probability. This is probably due to some technical problems during the reconstitution procedures, but it is of note that the incomplete restoration is in sharp contrast with the strictly all-or-none type effect of Ca^{2+} observed for the same centers as discussed above.

Similar extent (37%) of reactivation of O_2 evolution was possible by simply suspending the washed particles in high concentration of Cl^-, in agreement with previous studies by us [14] and others [20,21]. However, simulation of the flash O_2 yield pattern required an extraordinary high miss factor either specifically for the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition (70%) or uniformly for other S-state transitions (30%). Of the two alternative simulations, the former seems more likely from our previous observation that removal of the 33 kDa protein inhibits the $S_3 \rightarrow S_4$ transition. Whichever the better fitting is, either simulation indicates that the reactivation of O_2 evolution by high concentration of Cl^- is not due to complete restoration of the normal S-state turnover mechanism, but is rather due to a spontaneous (low probability) transition from S_3 to S_4. These observations and considerations lead us to the following view: the 33 kDa protein is not always indispensable for the $S_3 \rightarrow S_4$ transition, but affords a high probability for this transition.

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