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Effect of the 33-kDa protein on the S-state transitions in photosynthetic oxygen evolution

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The effect of the extrinsic 33-kDa protein on the photosynthetic oxygen evolution was studied by comparing spinach Photosystem II particles depleted of the 33-kDa protein with those reconstituted with the protein. The light-intensity dependence of the oxygen-evolution activity under continuous illumination suggests that a dark step, but not a light step, in the oxygen-evolving reaction is accelerated by the 33-kDa protein. Consistently, the pattern of oxygen yield with a series of short saturating flashes, which showed a maximum on the third flash and a damped oscillation with a period of 4, was not much affected by the removal and rebinding of the 33-kDa protein, when the dark interval between the flashes was long enough, i.e., longer than 0.5 s. The millisecond kinetics of oxygen release after the third flash was retarded by the removal of the 33-kDa protein and stimulated by its rebinding, suggesting that the transition from S_3 to S_0 is accelerated by the 33-kDa protein. The stability of the S_2 and S_3 states in darkness was higher in the absence of the 33-kDa protein than its presence.

Introduction

Recent biochemical studies on Photosystem II preparations from higher plants have revealed that three extrinsic proteins of 18 kDa, 24 kDa and 33 kDa participate in photosynthetic oxygen evolution [1–3]. The oxygen-evolving complex contains one molecule each of the three proteins and four Mn atoms per photochemical reaction center II [4]. The 18-kDa protein reduces the requirement for Cl^-, an essential factor for oxygen evolution [5,6]. The 24-kDa protein preserves the tight trap of Ca^{2+} in the oxygen-evolving complex [7,8]. The oxygen-evolving complex depleted of the 18-kDa and 24-kDa proteins requires 30 mM Cl^- and 5 mM Ca^{2+} for full oxygen-evolution activity [6,9,10].

The mode of action of Cl^- and Ca^{2+} on the transitions of S states in the Kok's scheme [11] has been investigated. Chloride ions are required for the transition from S_2 to S_3 [12,13]. The site of Ca^{2+} action is a subject of argument. Boussac et al. [14] inferred that Ca^{2+} is required for the transition from S_3 to S_0, whereas two groups reported that Ca^{2+} is necessary for the functional coupling between the photochemical reaction and

Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; Q_A, the primary quinone electron acceptor of Photosystem II; P-680, the primary electron donor of Photosystem II; Z, the secondary electron donor of Photosystem II.

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the oxidation of water [15,16]: according to Dekker et al. [15], the Ca^{2+} depletion uncouples the reaction between Z and the Mn-cluster in spinach PS II particles, and Satoh and Katoh [16] reported that the uncoupling takes place between Z and P-680 in cyanobacterial PS II particles.

The 33-kDa protein is necessary to preserve the binding of two of the four Mn atoms to the oxygen-evolving complex [17–19]. In addition, this protein seems to maintain the conformation of the Mn-cluster required for oxygen evolution: the oxygen-evolving complex depleted of the 33-kDa protein is inactive in oxygen evolution in 30 mM Cl^- even if all the Mn atoms remain bound to the complex [6]. Chloride ion at concentrations higher than 100 mM can partially be substituted for the 33-kDa protein in the preservation of the Mn binding and the oxygen-evolution activity [17,18].

In the present study, we investigated the effect of the 33-kDa protein on the S-state transitions using PS II particles from spinach thylakoids and found that the transition from S_3 to S_0 was retarded by the removal of the 33-kDa protein.

Materials and Methods

Preparation of PS II particles and the 33-kDa protein

PS II particles were prepared from spinach thylakoids with Triton X-100 according to the method of Kuwabara and Murata [1] and stored at −196°C in the presence of 30% (v/v) ethylene glycol [20]. Before use, the particles were washed three times with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) by centrifugation and suspension, and finally suspended in the same medium and then kept in darkness for 2 h.

PS II particles depleted of the 24-kDa and 18-kDa proteins were prepared by treating the PS II particles with 1.0 M NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) for 30 min under room light [20]. PS II particles depleted of all three extrinsic proteins were prepared by treating the PS II particles with 2.6 M urea/200 mM NaCl/25 mM Mes-NaOH (pH 6.5) for 30 min in darkness [17]. The treated particles were collected by centrifugation at 35 000 × *g* for 20 min and washed once with 200 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (hereinafter designated as high-salt medium) by resuspension and recentrifugation. The resultant pellets of the NaCl-treated and the (urea + NaCl)-treated particles were suspended in the high-salt medium containing 30% (v/v) ethylene glycol, unless otherwise stated, frozen at −196°C and stored at −196 or −80°C until use. Chl concentration was determined according to Arnon [21].

The 33-kDa protein, together with the 24-kDa and 18-kDa proteins, was extracted from untreated particles with 1.0 M Tris-HCl (pH 9.3 at 4°C)/300 mM sucrose and purified by column chromatography on DEAE-Toyopearl 650 M (Toyosoda) as described previously [22]. The obtained preparation of the 33-kDa protein was dialyzed against 10 mM Mes-NaOH (pH 6.5). No impurity of the preparation was detected by SDS-urea polyacrylamide gel electrophoresis [23]. The concentration of the 33-kDa protein was determined using its molar absorption coefficient of 20 $mM^{-1} \cdot cm^{-1}$ at 276 nm [24].

Rebinding of the 33-kDa protein to the (urea + NaCl)-treated particles was performed by adding the purified protein preparation to the particle suspension at a Chl concentration of 1 mg/ml and a protein-to-Chl ratio of 0.3 (w/w) which corresponds to two molecules of the protein per one oxygen-evolving complex [4]. After being kept in darkness for 30 min, the particles were collected by centrifugation at 35 000 × *g* for 20 min and resuspended in the high-salt medium containing 30% (v/v) ethylene glycol and kept frozen.

All the above procedures were performed at 0–4°C, and all handling of the (urea + NaCl)-treated particles was done under dim green light.

Measurement of oxygen evolution

Oxygen-evolution activity under continuous illumination was measured at 25°C using a Clark-type oxygen electrode in the presence of 0.05% bovine serum albumin and 0.3 mM phenyl-*p*-benzoquinone as an electron acceptor [1].

Oxygen evolution induced by a train of flashes was measured at 20°C in the absence of artificial electron acceptors using a Joliot-type oxygen rate electrode [25] equipped with a programmed flash illumination system as described previously [14]. The flow medium was 180 mM KCl/10 mM $CaCl_2$/25 mM Mes-NaOH (pH 6.5). PS II par-

ticles were suspended at 1.0 mg Chl/ml in 180 mM KCl/10 mM $CaCl_2$/25 mM Mes-NaOH (pH 6.5) containing 0.25 mg/ml catalase (bovine liver, Sigma). After a dark incubation for 30 min at 0°C, the suspension was placed on the polarized electrode and kept in darkness for 10–20 min. Xenon flashes of 2 μs spaced by 1.0 s were fired and the signals from the electrode were recorded after electronic differentiation [14]. The heights of the obtained spikes were taken as being proportional to the oxygen yield.

The turnover kinetics of the PS II reaction center following a charge separation was studied with the flash oxygen yield by a technique using two successive flashes spaced by a variable dark interval in the flash train [11,26]. The deactivation of the S states in darkness was measured by a method similar to the above [27,28]. PS II particles used for the measurements with the rate electrode were kept frozen in the absence of ethylene glycol, since its presence prevented sedimentation of the particles onto the platinum electrode.

For the measurement of the kinetics of oxygen release upon flash illumination, PS II particles were fixed on a platinum electrode as a thin layer by centrifugation (J. Lavorel, unpublished data). PS II particles were incubated at 1.0 mg Chl/ml with 0.25 mg/ml catalase in the high-salt medium for 30 min at 0°C in darkness. 15 μl of the particle suspension was diluted with 2 ml of 180 mM KCl/10 mM $CaCl_2$/25 mM Mes-NaOH (pH 6.5) and the particles were sedimented onto a flat and circular platinum electrode by centrifugation at $30000 \times g$ for 15 min in a swing rotor. The electrode together with the particle pellet and supernatant was mounted in a cuvette connected with a polarizer. After standing in the dark for 10–15 min at 20°C, three flashes spaced by 1.0 s were fired and the signal after the third flash was recorded.

Measurement of delayed fluorescence

Flash-induced delayed fluorescence was measured at 20°C using a stopped-flow fluorimeter described previously [29]. Excitation light from a 2-μs xenon flash was passed through an optical filter (Corning C-S 4-96), and the delayed fluorescence was detected through optical filters (Wratten 92 and Corning C-S 2-64) by a photomultiplier which had been gated off during the flash [14]. The particles were suspended in the high-salt medium containing 10 mM $CaCl_2$ at 10 μg Chl/ml and kept in darkness for 10 min at 20°C. After addition of 50 μM 2,5-dichloro-*p*-benzoquinone to the particle suspension three flashes spaced by 1.0 s were fired and the delayed fluorescence from 1.0 ms after each flash was detected. Eight signals from each fresh sample were accumulated and recorded.

Results

Treatment of PS II particles with 1.0 M NaCl specifically removed the 24-kDa and 18-kDa proteins and treatment with 2.6 M urea and 200 mM NaCl removed all the three extrinsic proteins as reported previously [17,20]. The NaCl-treated particles containing the 33-kDa protein were fully active in oxygen evolution in the presence of 5 mM Ca^{2+} and 30 mM Cl^- [6,10], whereas the (urea + NaCl)-treated particles depleted of the 33-kDa protein required 5 mM Ca^{2+} and 200 mM Cl^- for the maximum oxygen-evolution activity which was much lower than that of the NaCl-treated particles [6,18]. In order to investigate the effect of the 33-kDa protein on the oxygen evolution apart from the change in Cl^- requirement by the elimination of the 33-kDa protein, all measurements described below were done in the presence of 200 mM Cl^- [6]. 5 or 10 mM Ca^{2+} was also added to the reaction medium in order to maximize the activity of PS II particles depleted of the 24-kDa protein [10,18].

The light-intensity dependence of oxygen-evolution activity with the artificial electron acceptor under continuous illumination is presented in Fig. 1A. Oxygen-evolution activity of the (urea + NaCl)-treated particles was less than 50% of that of the NaCl-treated particles at the highest light intensity as previously reported [17], while practically no difference in the activity was observed at a low light intensity (Fig. 1A). Rebinding of the 33-kDa protein to the (urea + NaCl)-treated particles restored the activity at high light intensities, giving a light-intensity dependence similar to that of the NaCl-treated particles. Plots of oxygen-evolution activity vs. activity divided by light intensity (Fig. 1B) gave straight

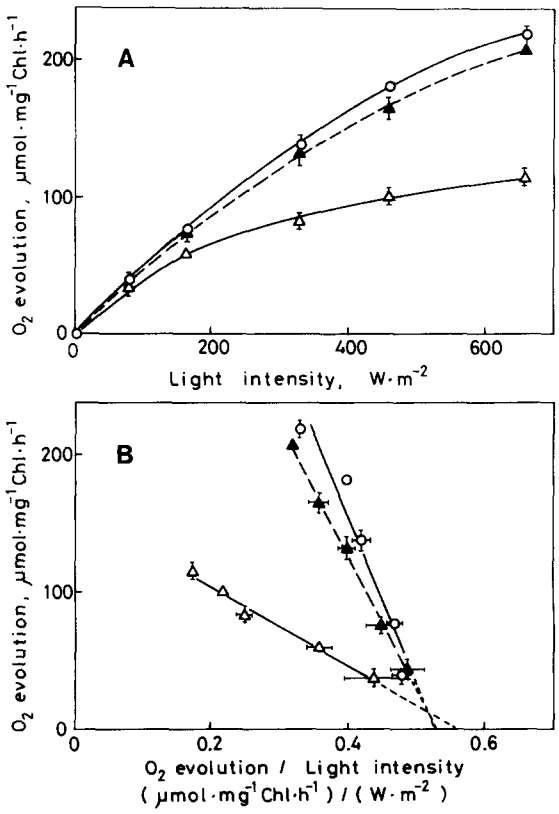

Fig. 1. Effect of the 33-kDa protein on the light-intensity dependence of oxygen evolution under continuous illumination. Oxygen-evolution activity was measured with phenyl-*p*-benzoquinone in 5 mM $CaCl_2$/200 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) and the light intensity was varied with neutral density filters. Averages of three measurements are presented and error bars represent the standard deviation. (A) Plot of oxygen-evolution activity vs. light intensity. (B) Plot of oxygen-evolution activity vs. activity divided by light intensity. O——O, NaCl-treated particles; △——△, (urea + NaCl)-treated particles; ▲------▲, (urea + NaCl)-treated particles supplemented with the 33-kDa protein at a protein-to-Chl ratio of 0.3 (w/w).

lines which intersected the abscissa at about the same points. If the plots are analyzed in a way similar to enzyme kinetics, the slope, abscissa intercept and ordinate intercept of the line correspond to a negative value for the apparent Michaelis constant ($-K'_m$), relative quantum yield and maximum rate, respectively [30–33]. The results in Fig. 1B indicate that the K'_m and the maximum rate were reduced by removal of the 33-kDa protein and recovered by its rebinding, while the relative quantum yield remained constant throughout. These findings suggest that almost all the oxygen-evolving complexes in the (urea + NaCl)-treated particles can evolve oxygen, and that a dark, but not a light, process is slowed down by the elimination of the 33-kDa protein.

However, in some cases in the course of the present study, the urea plus NaCl treatment decreased the relative quantum yield of oxygen evolution by 30%. In these cases rebinding of the 33-kDa protein did not restore the relative quantum yield. These observations suggest that the urea plus NaCl treatment sometimes causes inactivation of a fraction of the oxygen-evolving complexes due to some irreversible damage other than the elimination of the 33-kDa protein, probably partial release of Mn.

Patterns of oxygen yield obtained by illumination with a series of short saturating flashes spaced by 1.0 s are presented in Fig. 2. Catalase was added to the particle suspensions in order to eliminate anomalous production of oxygen which was caused by hydrogen peroxide and weakly bound Mn present in the preparations [34,35]. The three types of particles showed a maximum on the third flash and a damped oscillation with a period of four. When the dark interval between the flashes was shorter than 0.5 s, however, the oxygen-yield pattern in the (urea + NaCl)-treated particles depleted of the 33-kDa protein deviated from the typical pattern as presented in Fig. 2 in such a way that the oxygen yield on the fourth flash became larger than that on the third flash (data not shown). On the other hand, in the particles containing the 33-kDa protein the oxygen yield pattern was not affected when the dark interval between the flashes was shortened to 0.2 s. These findings suggest that all the S-state transitions in the PS II particles depleted of the 33-kDa protein proceeded properly under flash illumination with a sufficient dark interval between the flashes.

In order to clarify the effect of the 33-kDa protein on the dark processes in detail, the turnover time of the PS II reaction center after a charge separation in relation to the S state was studied by measuring the oxygen yield on the third flash when a dark interval between two successive flashes in a flash train was varied. Table I shows that the turnover of the reaction center either at the S_1 or S_2 state was not affected by the removal

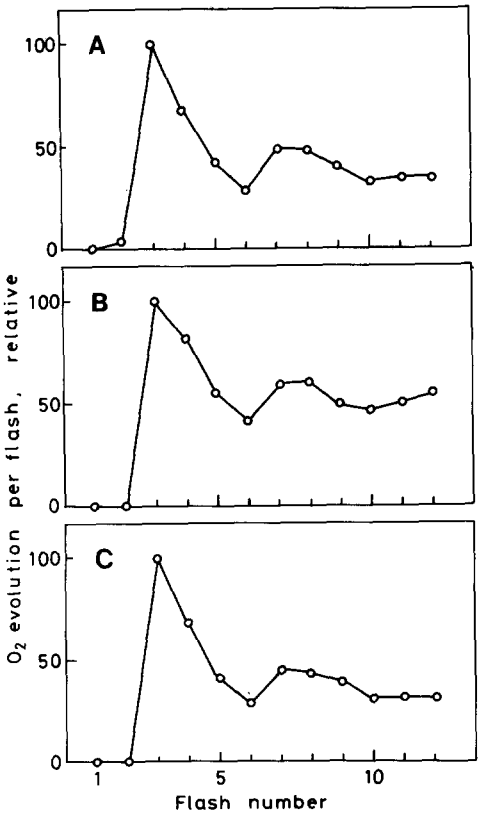

Fig. 2. Effect of the 33-kDa protein on the pattern of flash-induced oxygen yield. Oxygen evolved per flash was measured using an oxygen rate electrode in the presence of catalase with the dark interval between flashes of 1.0 s. Particles were kept on the electrode in darkness for 10 min, except for the (urea + NaCl)-treated particles which were kept for 20 min in darkness. The data are normalized to the yield on the third flash. (A) NaCl-treated particles. (B) (Urea + NaCl)-treated particles. (C) (Urea + NaCl)-treated particles supplemented with the 33-kDa protein at a protein-to-Chl ratio of 0.3 (w/w).

and rebinding of the 33-kDa protein. Probably, this suggests that the 33-kDa protein does not affect the transitions from S_1 to S_2 and from S_2 to S_3. However, this is not very conclusive, since the turnover time of the PS II reaction center thus measured could depend on the re-oxidation of Q_A^-, if it is slower than the re-reduction of Z^+, that is, the transition of S state [11,26]. This possibility can not be excluded.

Table I and Fig. 3 show the stability of the S_2 and S_3 states in darkness. In the NaCl-treated particles both S states had about the same half-times of deactivation, about 30 s, which were

TABLE I

THE EFFECT OF THE 33-kDa PROTEIN ON THE TURNOVER OF PHOTOSYSTEM II REACTION CENTER AND ON THE DEACTIVATION OF THE S STATE

The turnover time of the reaction center at the S_1 state and the deactivation time of the S_2 state were studied by measuring the oxygen yield on the third flash upon varying a dark interval between the first and second flashes in a train of flashes spaced by 1.0 s. The turnover time at the S_2 state and the deactivation time of the S_3 state were studied by measuring the oxygen yield on the third flash on varying a dark interval between the second and third flashes. Catalase was present during the measurements. Values represent the time required for 50% of the oxygen-evolving complexes to undergo turnover or deactivation.

Type of particles	Turnover ($t_{1/2}$, ms)		Deactivation ($t_{1/2}$, s)	
	$S_1(\rightarrow S_2)$	$S_2(\rightarrow S_3)$	S_2	S_3
NaCl-treated	6	1.5	34	28
(Urea + NaCl)-treated	5	2.2	160	40
(Urea + NaCl)-treated + 33-kDa protein	5	1.5	12	13

slightly shorter than those in spinach thylakoids [27,28]. The elimination of the 33-kDa protein upon the urea plus NaCl treatment remarkably stabilized the S_2 state to increase the half-time of deactivation to 160 s, while the S_3 state was affected only slightly. The rebinding of the 33-kDa protein accelerated the deactivation of the S_2 and S_3 states. These observations suggest that the properties of the S_2 state are modified by the 33-kDa protein.

The effect of the 33-kDa protein on the transition from S_3 to S_0 was studied by measuring fast kinetics of oxygen release after the third flash (Fig. 4). For this measurement the particles were sedimented onto a surface of the platinum electrode as a thin layer by centrifugation in order to raise the response of the electrode. In all three types of particles, a fast rise to the maximum level occurred after the flash and then a slow decay followed. It is obvious that the oxygen release was retarded by the elimination of the 33-kDa protein and accelerated by its rebinding to exhibit a shape similar to the NaCl-treated particles. In this method the shape of the trace is supposed to be affected not only by the kinetics of oxygen release

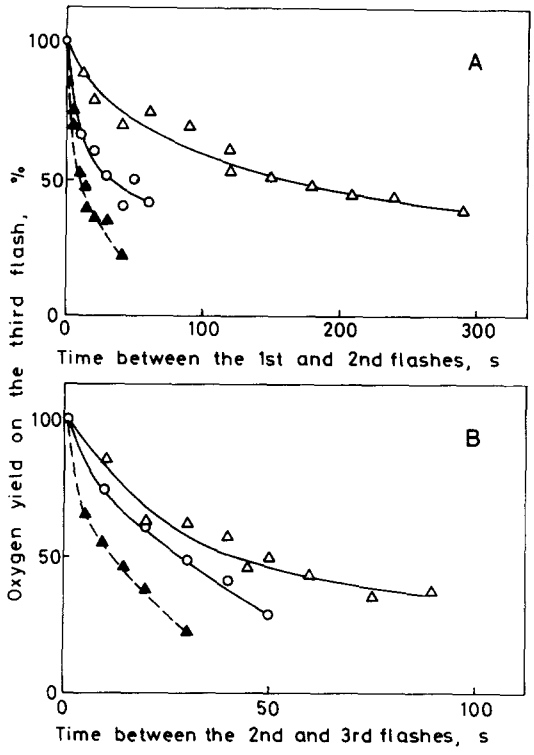

Fig. 3. Effect of the 33-kDa protein on the kinetics of S-state deactivation. PS II particles which had been incubated with catalase were placed on an oxygen rate electrode and kept in darkness for 10 min. The oxygen yield on the third flash was measured with various dark intervals between two successive flashes in the flash train as described in the legend for Table I. 100% corresponds to the oxygen yield on the third flash in a flash train spaced by 1.0 s. (A) Deactivation kinetics of the S_2 state. (B) Deactivation kinetics of the S_3 state. O———O, NaCl-treated particles; Δ———Δ, (urea + NaCl)-treated particles; ▲-----▲, (urea + NaCl)-treated particles supplemented with the 33-kDa protein at a protein-to-Chl ratio of 0.3 (w/w).

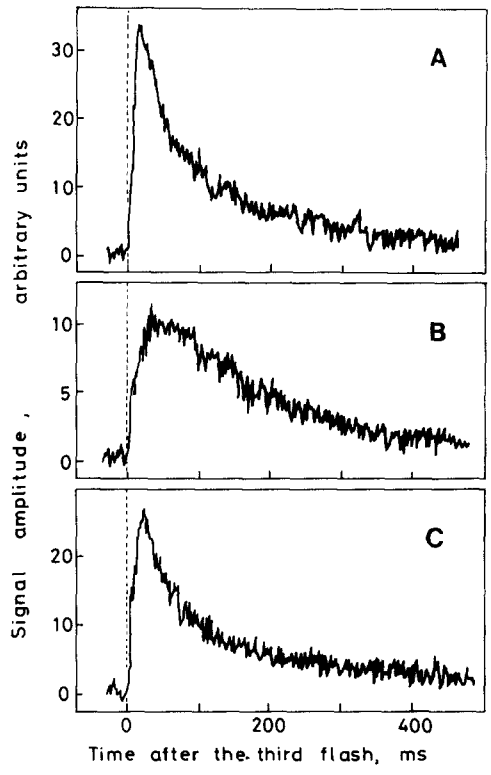

Fig. 4. Effect of the 33-kDa protein on the fast kinetics of oxygen release after the third flash. Particles which had been incubated with catalase were sedimented onto the platinum electrode by centrifugation. The signal after the third flash was recorded after amplification but with no differentiation. (A) NaCl-treated particles. (B) (Urea + NaCl)-treated particles. (C) (Urea + NaCl)-treated particles supplemented with the 33-kDa protein at a protein-to-Chl ratio of 0.3 (w/w).

from the oxygen-evolving complex, but also by other factors such as the thickness of the particle layer and the diffusion coefficient of oxygen molecules through the layer. We observed that when the thickness of the particle layer was decreased, the decay after the maximum was accelerated, but the fast rise was not significantly affected (data not shown). This observation suggests that the rise kinetics reflects the formation and liberation of oxygen molecules in the particles which were adjacent to the electrode surface. Although the correlation between the rise time of the signal and the time of oxygen release may not be exactly linear, it can be concluded from the time to reach the maximum level (Fig. 4) that the removal of the 33-kDa protein retarded the oxygen release from the oxygen-evolving complex approx. 2.5 times.

Fig. 5 shows the effect of the rebinding of the 33-kDa protein on the decay of delayed fluorescence up to 13 ms after the first, second and third flashes. Particles containing and lacking the 33-kDa protein exhibited similar decay curves after either the first or second flash (Figs. 5A and B), whereas the decay of delayed fluorescence after the third flash was markedly stimulated by rebinding of the 33-kDa protein (Fig. 5C). Since the delayed fluorescence originates from a charge recombination between positive and negative charges located at the donor and acceptor sides, respec-

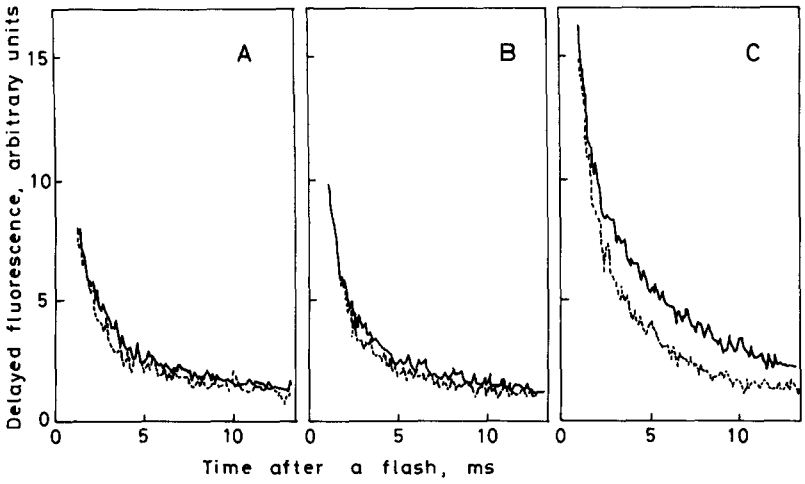

Fig. 5. Effect of the 33-kDa protein on the decay of the delayed fluorescence after the first, second and third flashes. Particle suspension was illuminated with three flashes spaced by 1.0 s. Delayed fluorescence was recorded from 1.0 ms after the first (A), the second (B), and the third (C) flashes. ———, (Urea + NaCl)-treated particles; - - - - - -, (urea + NaCl)-treated particles supplemented with the 33-kDa protein at a protein-to-Chl ratio of 0.3 (w/w).

tively, of the PS II reaction center [36], its decay represents the disappearance of the charge pairs in PS II. In the time range from 1 to 13 ms after the third flash the most probable candidate for the charge at the donor side of PS II is the state of S_3Z^+. Therefore, the observation that the depletion of the 33-kDa protein slowed down the decay of delayed fluorescence (Fig. 5C) may indicate that the 33-kDa protein accelerated the transition from the S_3Z^+ to S_4Z state.

Discussion

Treatment of PS II particles with 1 M $CaCl_2$ [37] or 2.6 M urea plus 200 mM NaCl [17] removes all the three extrinsic proteins from the oxygen-evolving complex leaving the four Mn atoms bound. The stoichiometric amount of the 33-kDa protein can rebind to the particles lacking the three proteins [18,38]. Therefore, the function of the 33-kDa protein can be investigated by comparing the particles depleted of the protein and those reconstituted with it.

The oxygen-evolution activity under continuous illumination at a saturating intensity of the (urea + NaCl)-treated particles was lower than that of the particles containing the 33-kDa protein. The light-intensity dependence of the oxygen-evolution activity (Fig. 1) suggests that a dark step is retarded by the elimination of the 33-kDa protein. The transitions from S_1 to S_2 and from S_2 to S_3 were not detectably affected by the removal and rebinding of the 33-kDa protein (Table I). The formation and release of oxygen molecules from the oxygen-evolving complex, that is, the transition from S_3 to S_0, was slowed down by the elimination of the protein and fully recovered by its rebinding. Therefore, it can be concluded that the suppression of the oxygen evolution under continuous illumination by depleting the 33-kDa protein is caused by the slowdown of the transition from S_3 to S_0.

Based on the present knowledge on the mechanism of oxygen evolution, the transition from S_3 to S_0 with concomitant release of an oxygen molecule is supposed to be composed of three steps: (1) the oxidation of the Mn cluster by Z^+ to convert S_3 to S_4; (2) the formation of an oxygen molecule concomitantly with the conversion from S_4 to S_0; (3) the release of the oxygen molecule from the complex. The results of the delayed fluorescence (Fig. 5) suggest that at least the first step, the transition from S_3 to S_4, is accelerated by the 33-kDa protein. Further study is required to determine whether the other steps are also accelerated by the 33-kDa protein.

In the study on the S-state transitions by thermoluminescence, Ono and Inoue [39] reported that the oxygen-evolving complex lacking the 33-kDa protein could undergo the S-state transitions up to the S_3 state, but not that from S_3 to S_0. This is apparently incompatible with our present result that this transition is only retarded by the elimination of the 33-kDa protein. The discrepancy may originate from the difference in the Cl^- concentration in the reaction medium. Ono and Inoue [39] used 20 mM Cl^-, which is not sufficient to sustain the oxygen evolution in the PS II particles depleted of the 33-kDa protein [6]. Therefore, in their study, they observed two effects on the S-state transition; one is the effect of Cl^- depletion because the removal of the 33-kDa protein dramatically increased the Cl^- requirement of oxygen evolution [6], and the other is the effect of the 33-kDa protein itself. Since a sufficient concentration of Cl^- was used in the present study, we could discriminate between the effect of the 33-kDa protein and that of the Cl^- depletion.

A striking feature of the oxygen-evolving complex lacking the 33-kDa protein is the pronouncedly slow deactivation of the S_2 state (Table I, Fig. 3), which is considered to arise from the back reaction in PS II [27,40–42]. The change in the deactivation rate upon removal and rebinding the 33-kDa protein may have resulted from the modification of the oxidation-reduction potential of the S states by the 33-kDa protein, which is supposed to interact with the Mn-cluster.

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