Biochimica et Biophysica Acta 892 (1987) 224–235
Elsevier

BBA 42556

Stability and oscillation properties of thermoluminescent charge pairs in the O_2-evolving system depleted of Cl^- or the 33 kDa extrinsic protein

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(Received 9 December 1986)

Key words: Oxygen evolution; Photosystem II; Protein, 33 kDa; Chloride effect; Thermoluminescence

The effects of Cl^- depletion and removal of the 33 kDa extrinsic protein on the charge stabilization in O_2-evolving Photosystem II (PS II) particles were studied by curve fitting and deconvolution of thermoluminescence bands. The following results were obtained. (1) Cl^- depletion reversibly decreases the redox potential of the S_2 state by 60–80 mV, and thereby elevates the recombination temperature of both $S_2Q_B^-$ and $S_2Q_A^-$ charge pairs. (2) Removal of the 33 kDa extrinsic protein specifically elevates the recombination temperature of the $S_2Q_A^-$ charge pair, with practically no effect on the $S_2Q_B^-$ pair. This was tentatively interpreted as showing that the protein removal decreases the redox potential of both S_2 and Q_B^-, but not of Q_A^-, and, thus, the effects are mutually cancelled for the $S_2Q_B^-$ pair, but are manifested for the $S_2Q_A^-$ pair. (3) Deconvolution of glow curves demonstrated that S_3 is not formed in Cl^--depleted PS II, but is formed in 33 kDa protein-depleted PS II even at a low (20 mM) Cl^- concentration. Analysis of thermoluminescence oscillations confirmed that Cl^- depletion interrupts S_2–S_3 transition, whereas the protein removal interrupts S_3–(S_4)–S_0 transition at 20 mM Cl^-. (4) Cl^- depletion by SO_4^{2-} replacement in the absence of 33 kDa protein affected thermoluminescence in a different way from that in the presence of the protein. Based on these findings, the properties of charge pairs in the Cl^--depleted PS II particles were discussed in relation to the role of the 33 kDa extrinsic protein.

Introduction

It has been established that functionally active Mn, Cl^- and the 33 kDa extrinsic proteins are involved in photosynthetic O_2 evolution (see reviews of Refs. 1–4). Recent biochemical techniques enable us to remove Cl^- [5,6] and the 33 kDa protein [7,8] from the O_2-evolving apparatus, while leaving the functional Mn atoms in the active center.

Cl^- depletion results in complete but reversible inactivation of O_2 evolution [5,6]. Regarding this inactivation, various inhibition sites have been proposed by different research groups: inhibitions of S_2–S_3, S_1–S_2 and S_3–(S_4)–S_0 transitions based on measurements of fluorescence quenching [9,10], EPR multiline signal [11,12], and oscillation of thermoluminescence [13], respectively. Ono et al. [14] found that upon illumination Cl^- depleted samples formed an S_2 state that was abnormal in the sense that it did not show a multiline EPR signal. Also, they found that this abnormal S_2

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Abbreviations: Chl, chlorophyll; DCMU (≡diuron), 3-(3,4-dichlorophenyl)-1,1′-dimethylurea: Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; Q_A and Q_B, primary and secondary quinone acceptors of PS II.

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state does not undergo further S-state transitions. The increased stability of the S_2 state in Cl^--depleted samples was suggested much earlier by Muallem et al. [15,16]. The Cl^--dependent reversibility of this abnormal S_2 is in good parallelism with the reversible appearance of the abnormal thermoluminescence band in Cl^--depleted PS II reported by Homann et al. [13], but there remains some inconsistency with regard to its oscillatory behavior. The first part of this study aims at a clarification of this problem.

Mn-preserving removal of the three extrinsic proteins by $CaCl_2$ wash [7] or urea/NaCl wash [8] also resulted in inactivation of O_2 evolution (at low Cl^- concentration) [7,8]. From the absence of the S_2-specific multiline EPR signal in the washed particles [17], it was once considered that the O_2-evolving enzyme did not undergo any S-state transition in the absence of the extrinsic proteins [11]. However, the inactivation was shown to be appreciably reversed by inclusion of a high concentration of Cl^- in the suspension medium [18,19] as well as by reconstitution with extracted 33 kDa extrinsic protein [20,21]. A part of this high Cl^- effect is to protect the Mn atoms from being released out of the washed particles [18,22], but it is also considered that the Cl^- demand (for O_2 evolution) is highly enhanced in the absence of the 33 kDa protein; and a high concentration of Cl^- is needed to meet this enhanced demand. It was reported that a high concentration of Cl^- restores the period-four oscillation of flash O_2 yield with retarded kinetics of O_2 evolution [23], or the multiline EPR signal accompanied by its quadruple oscillation [24].

If we assume the above hypothesis of an enhanced Cl^- demand due to the absence of the 33 kDa protein, it may be expected that removal of the extrinsic proteins would result in Cl^--depleted conditions. However, Ono and Inoue [25] reported that the O_2-evolving enzyme in $CaCl_2$-washed particles suspended in 20 mM Cl^- undergoes S-state transitions up to the S_3 state, but not beyond S_3. It is difficult to reconcile this observation with the most plausible effect of Cl^- depletion, the inhibition of S_2–S_3 transition [9,10,14]. The second part of this study aims at a clarification of these problems.

To solve these problems, we used thermoluminescence measurements throughout this study. By means of curve fitting of glow peaks, the thermoluminescence components superimposed on each other were deconvoluted, and we were able to show clearly that S_3 is not formed in Cl^--depleted particles, but is formed in the 33 (and 24 and 16) kDa proteins(s)-depleted particles. Some of the characteristics of the glow curves of the extrinsic protein-depleted PS II particles suspended in high salt concentrations were also studied.

Materials and Methods

Oxygen-evolving Triton PS II particles were prepared from spinach as described in Ref. 25, and stored at liquid N_2 until use. After thawing, the particles were washed twice with 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5), and subjected to further treatments and measurements.

Mn-preserving removal of the 16, 23 and 33 kDa extrinsic proteins was attained by treating the PS II particles for 30 min either with 1.5 M $CaCl_2$/400 mM sucrose/40 mM Mes-NaOH (pH 6.5) [7], or with 2.7 M urea/400 mM sucrose/200 mM NaCl/40 mM Mes-NaOH (pH 6.5) [8]. The treated membranes were washed twice with 400 mM sucrose/200 mM NaCl/4 mM Mes-NaOH (pH 6.5) and resuspended in the same medium. All procedures were done at 0 °C under dim green light.

Reconstitution with 33 kDa extrinsic protein was done as described in Ref. 20. The $CaCl_2$- or urea/NaCl-washed particles were incubated with isolated 33 kDa extrinsic protein in 400 mM sucrose/200 mM NaCl/4 mM Mes-NaOH (pH 6.5) at 0 °C for 30 min.

Cl^- depletion was attained by replacement of Cl^- with SO_4^{2-} as described in Ref. 13. The PS II particles were suspended in 400 mM sucrose/1 mM NaCl/4 mM Mes-NaOH (pH 6.5) after two washes with the same medium, diluted (10-fold) with 400 mM sucrose/70 mM Na_2SO_4/40 mM Mes-NaOH (pH 7.5) and then incubated at 0 °C for 5 min in darkness.

Thermoluminescence was measured as described previously [26]. The samples (about 20 μg Chl/80 μl) were exited with 5 μs xenon flashes at

saturating intensity and glow curves were recorded at a heating rate of about 0.7 °C/s. The heating rate slightly changed during heating. The thermoluminescence intensity and the heating rate were digitized as a function of sample temperature, using a Hewlett-Packard microcomputer (9825A) equipped with a Nicolet digital oscilloscope (Explorer IIIA), and curve fitting, deconvolution and calculations of activation free energies were done with a Hewlett-Packard microcomputer (9826) as in Ref. 27, taking into account the nonlinear heating rate. Theoretical functions for photosynthetic charge recombinations were assumed as in Refs. 27, 28. The lifetime of thermoluminescent charge pairs was determined by plotting the total luminescence as a function of incubation time (at 25 °C) placed between flash excitation and cooling. The total luminescence was estimated from the area under a glow peak.

Results and Discussion

Effects of Cl^- depletion and removal of the extrinsic proteins on the stability of $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs

Fig. 1 shows the single-flash-induced glow curves of dark-adapted PS II particles. In control PS II particles, the B-band originating from the recombination of the $S_2Q_B^-$ charge pair [26,29] was observed at around 30 °C (curve a). When Q_A to Q_B electron transfer was blocked, the B-band was converted to the Q-band, emitting at around 10 °C (curve b). The Q-band originates from the $S_2Q_A^-$ charge recombination [26,29]. (For a recent review on the origin of various thermoluminescence bands, see Sane and Rutherford [30].) Both of these curves could be satisfactorily fitted by assuming a respective single theoretical component for each curve, although some minor components had to be assumed for better fitting. The 10 °C component in the B-band is probably due to a minor subpopulation of centers in which Q_B is disconnected from Q_A. In turn, the 30 °C component in the Q-band may be due to the subpopulation not affected by DCMU (≡diuron).

Single-flash excitation of Cl^--depleted samples also induced the B- and Q-bands (curves c, d). However, these bands were markedly different from those in control samples: higher intensity and higher peak temperature with narrower band width, as was shown earlier by Homann et al. [13]. These changes induced by Cl^- depletion were almost completely reversed by the addition of 50 mM Cl^- (curves e, f), although the increase in band height was not always completely reversed. These glow curves could also be fitted by assuming a respective single theoretical component for each, even though some minor components had to be assumed.

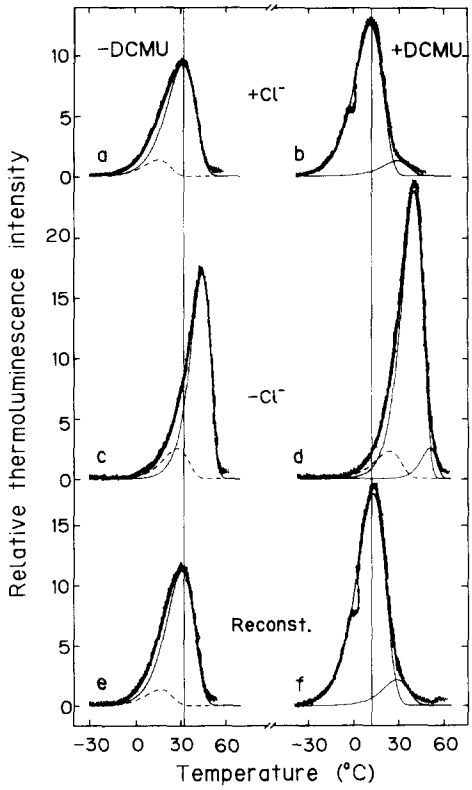

Fig. 1. Effect of Cl^- depletion on thermoluminescence glow curves of PS II particles (pH 7.5). Dark-adapted samples were excited by a single flash at 20 and −5 °C for measurements of B-band (a, c, e) and Q-band (b, d, f) in the absence and presence of 10 μM DCMU (diuron), respectively. (a, b) Untreated control in 20 mM NaCl; (c, d) Cl^--depleted by replacement with 70 mM Na_2SO_4; (e, f) Cl^--reconstituted by inclusion of 50 mM NaCl in samples for c, d. Solid and broken thin curves are the components assumed by curve fitting.

Fig. 2 shows the single-flash-induced glow curves of the PS II particles depleted of all the three extrinsic proteins either by $CaCl_2$ wash or by urea/NaCl wash. In the washed samples the peak

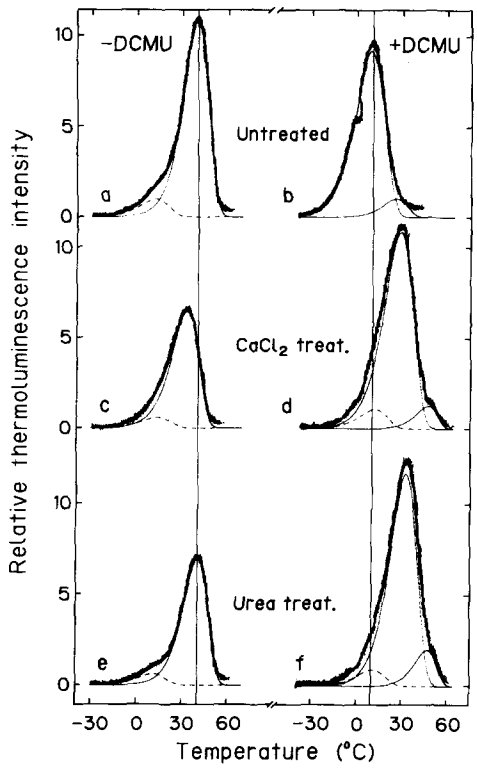

Fig. 2. Effect of removal of extrinsic proteins on thermoluminescence glow curves of PS II particles (pH 5.5). Dark-adapted samples were excited by a single flash at 20 and −5 °C for measurements of B-band (a, c, e) and Q-band (b, d, f) in the absence and presence of 10 μM DCMU (≡diuron), respectively. (a, b) Untreated control; (c, d) the extrinsic proteins were removed by $CaCl_2$ treatment; (e, f) the extrinsic proteins were removed by urea/NaCl treatment. All samples contained 20 mM NaCl. Solid and broken thin curves are the components assumed by curve fitting.

temperature of the Q-band was upshifted by about 25 °C (curves d, f) relative to the control (curve b). In contrast, the peak temperature of the B-band was not affected at all by the urea/NaCl wash (curve e), or was slightly shifted to lower temperatures by $CaCl_2$ wash (curve c). These results show that the effects on thermoluminescence of the removal of the extrinsic proteins are very different from those of Cl^- depletion and the $S_2Q_A^-$ charge pair is preferentially stabilized (Q-band upshifted in temperature) with less effect on the $S_2Q_B^-$ pair. The stabilization effect of the $S_2Q_A^-$ charge pair does not require the presence of DCMU or other inhibitors and can be completely reversed by the rebinding of the 33 kDa extrinsic protein, indicative of absolute specificity for the absence of the 33 kDa protein [31]. All these glow curves could also be satisfactorily fitted by assuming a respective single theoretical component for each, although, again, some minor components had to be assumed for complete fitting.

Table I summarizes the energetic parameters calculated from the individual theoretical components assumed for fitting of the glow curves shown in Figs. 1 and 2. By Cl^- depletion, the stabilities of both the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs were increased by 55 and 80 meV, respectively, in terms of activation free energy (ΔG^*). The lifetimes of these charge pairs predicted from the calculated ΔG^* values were in good agreement with those directly measured. In contrast to a reasonably parallel increase in stabilization of $S_2Q_A^-$ and $S_2Q_B^-$ pairs induced by Cl^- depletion, the removal of the 33 kDa extrinsic protein induced a quite uneven increase in stabilization between the two charge pairs: the $S_2Q_B^-$ pair was slightly destabilized by the $CaCl_2$ wash but practically unchanged by the urea/NaCl wash, whereas the stability of the $S_2Q_A^-$ pair was increased by 50–55 meV, accompanied by an approx. 10-fold increase in lifetime. The control ΔG^* values for Cl^- depletion are not the same as those for 33 kDa protein removal. This is due to the difference in pH, 7.5 for Cl^- depletion but 5.5 for 33 kDa protein removal: peak temperature of the $S_2Q_B^-$ pair is elevated at lower pH values, due to protonation of Q_B^-, while that of $S_2Q_A^-$ is not, since Q_A^- is not protonated [22].

The parallel increase in stabilization of $S_2Q_B^-$ and $S_2Q_A^-$ by Cl^- depletion can be explained by assuming that Cl^- depletion affects largely the stabilization conditions of S_2 with little or no effect on Q_B^- and Q_A^- [33]: formation of an abnormal S_2 with a lowered (by 50–80 mV) redox potential. From its reversible characteristics, dependent on Cl^- concentration, this abnormal S_2 can be correlated with the EPR multiline signal-silent S_2 reported by Ono et al. [14]. On the other hand, the $S_2Q_A^-$-specific stabilization by removal of the extrinsic proteins is difficult to explain. Although at present, we cannot provide a decisive mechanism for the specificity, the following two alternatives are suggested. (i) The stabilization conditions of Q_A^- are specifically affected, but

TABLE I

EFFECTS OF Cl^- DEPLETION AND REMOVAL OF THE 33 kDa EXTRINSIC PROTEIN ON THE ACTIVATION FREE ENERGY (ΔG^*) AND THE HALF-LIFE ($t_{1/2}$) OF THE $S_2Q_B^-$ AND $S_2Q_A^-$ CHARGE PAIRS

Charge pair (condition)	ΔG^* (meV)	ΔG^* (treat) − ΔG^* (cont) [a] (meV)	$t_{1/2}$ (s)	
			calculated	measured
Cl^- depletion (pH 7.5)				
$S_2Q_B^-$ control	840 ± 4	-	18 ± 5	25
$-Cl^-$	894 ± 6	54 ± 6	150 ± 33	138
$S_2Q_A^-$ control	800 ± 4	-	3.8 ± 0.4	3
$-Cl^-$	878 ± 5	78 ± 5	78 ± 15	92
Removal of 33 kDa protein (pH 5.5)				
$S_2Q_B^-$ control	869 ± 10	-	56 ± 2	63
$CaCl_2$ wash	855 ± 7	−14 ± 8	32 ± 7	43
urea wash	871 ± 5	2 ± 7	59 ± 11	60
$S_2Q_A^-$ control	792 ± 2	-	2.5 ± 0.3	3
$CaCl_2$ wash	842 ± 6	50 ± 4	21 ± 4	30
urea wash	848 ± 3	56 ± 3	25 ± 2	36

[a] ΔG^* (treat) − ΔG^* (cont) stands for the change in activation free energy (meV) induced by Cl^- depletion and removal of the 33 kDa protein.

those of Q_B^- and S_2 are not. (ii) The stabilization conditions of Q_B^- and S_2 are largely affected, with little or no effect on Q_A^-, and the effects on Q_B^- and S_2 compensate with each other to fortuitously cancel the changes in energetic parameters (e.g., similar extent of decrease in redox potentials of Q_B^- and S_2). The latter interpretation seems more likely, because the $CaCl_2$-washed PS II membranes (at low Cl^- concentration) do not show the multiline EPR signal [11,17], indicative of the formation of modified S_2, and also because the affinity to artificial acceptor quinones is much altered after $CaCl_2$ wash [34], indicative of some effect on Q_B and Q_A. Possibly, Q_A is less affected than Q_B due to its buried environments, which would result in an $S_2Q_A^-$-specific effect.

S-state turnovers in the absence of Cl^-

In normal PS II particles with an active O_2-evolving system, the B-band intensity shows a period-four oscillation as a function of flash number. The oscillation pattern shows maxima after the first and fifth flashes in well dark-adapted particles with an initial distribution of $Q_B : Q_B^- = 75 : 25$ [26,29], and a distortion or abolition of this pattern implies an inhibition of the S-state transition(s). As reported in Ref. 13, Cl^- depletion of dark-adapted particles results in an interruption of the B-band oscillation after the second flash, and this phenomenon was interpreted as indicating that the S_3–(S_4)–S_0 transition was inhibited by Cl^- depletion. This interpretation is not consistent with the fluorescence data that Cl^- depletion inhibits the S_2–S_3 transition [9,10]. We attempted to clarify this problem by means of glow curve deconvolution.

As shown in Fig. 3 (left panel), the glow curve after the first flash excitation of Cl^--sufficient normal particles consisted of a single component peaking at 40 °C accompanied by a negligibly small component at 10 °C (broken curve). After the second flash, the amplitude of the 40 °C component was decreased to about one-third, and a new component appeared at 28 °C. These two components correspond to the so-called B_2 (40 °C) and B_1 (28 °C) bands originating from $S_2Q_B^-$ and $S_3Q_B^-$ recombinations, respectively [35]. It is of note that these two bands can be separated only at low pH values [36]. The glow curves after the third and fourth flashes were also deconvoluted into two components, and the relative amplitude of the two components changed depending on the number of flashes. These changes in the amplitudes of the two bands are consistent with the proposed

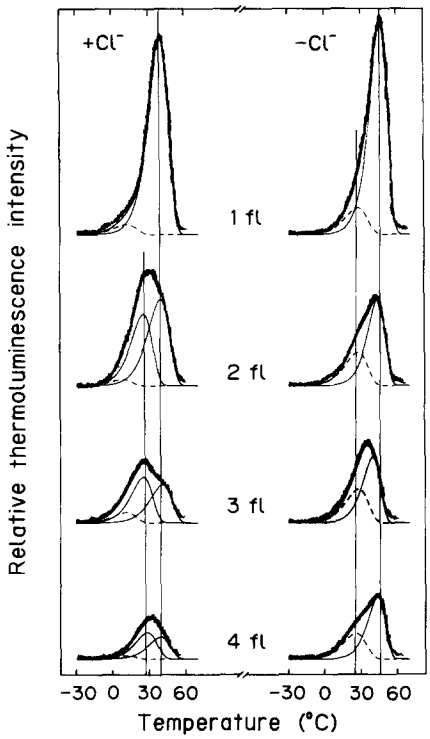

Fig. 3. Deconvolution of glow curves of Cl^--sufficient and Cl^--depleted PS II particles (pH 6.0). Dark-adapted samples were excited by one to four flashes (fl) at 20 °C. Left panel, Cl^--sufficient control in 20 mM NaCl; Right panel, Cl^--depleted by alkaline shock as described in Ref. 13. Peak heights of the glow curves were normalized on the basis of those after the first flash. Solid and broken thin curves are the components assumed by curve fitting.

mechanism of thermoluminescence oscillation [26,29], and verify the deconvolution technique.

The right panel of Fig. 3 shows the deconvolution of glow curves of Cl^--depleted particles. The abnormal B-band peaking at 45 °C induced by the first flash could be fitted mostly by a single component, but a minor component had to be assumed at around 26 °C (broken curve) for complete fitting. This minor component is probably due to irreversibly (non-specifically) inactivated centers, since its amplitude neither decreased nor increased after repeated excitation. After the second flash, the 45 °C component arising from the abnormal $S_2Q_B^-$ charge pair decreased to the same extent as in the Cl^--sufficient control, but no new component could be found other than the above-mentioned minor component. These components underwent practically no further change upon increasing the flash number.

The absence of a new band corresponding to $S_3Q_B^-$ after the second or further flashes suggested that the S_3 state was not formed in the absence of Cl^-. However, the fact that the 45 °C component was markedly decreased after the second flash indicates that the abnormal $S_2Q_B^-$ pairs were partially lost, or converted to some other charge pair(s) by the second flash. Ono et al. [37] have proposed an explanation for this phenomenon: they assumed that one electron can be transferred to the acceptor side from the abnormal $S_2Q_B^-$ centers (probably from Z, the secondary donor of PS II) without involving any S-state transition, which results in a non-thermoluminescent S_2Q_B pair (via $S_2Q_B^{2-}$). This interpretation is consistent with the earlier findings by Itoh et al. [9] and Theg et al. [10] that in Cl^--depleted samples S-state transitions stopped at S_2, but two electrons could be transferred through PS II; one provided by S_1–S_2 transition and the other by Z–Z^+ transition on the first and second flashes, respectively.

Such confusion arising from the acceptor side-effect can be minimized if we control the initial $Q_B : Q_B^-$ ratio to be 50 : 50 by a brief continuous preillumination of the sample followed by short dark adaptation, since in these samples the $Q_B : Q_B^-$ ratio keeps a constant level independent of the flash number [29,38]. Fig. 4 shows the glow curves measured under such conditions. The Cl^--sufficient particles showed a period-four oscillation with maxima after the second and sixth flash (instead of maxima after the first and fifth flash in PS II particles with $Q_B : Q_B^- = 75 : 25$ shown in Fig. 3), while in Cl^--depleted particles, the abnormal $S_2Q_B^-$ band formed by the first flash did not change after the second and further flash excitations. These results indicate that the S-state transition does not proceed beyond S_2 in the absence of Cl^-, and supports the conclusion obtained by deconvolution experiments in Fig. 3.

S-state turnovers in the absence of the extrinsic proteins

Fig. 5 shows the deconvolution of the glow curves of PS II particles depleted of the three extrinsic proteins. For a better resolution of the

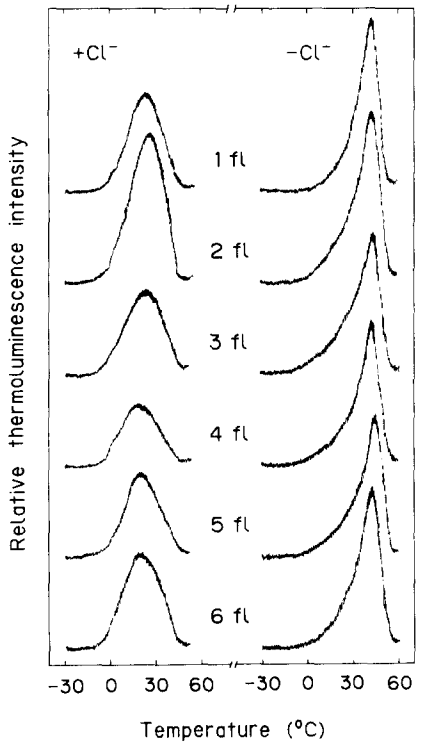

Fig. 4. Oscillations of glow curves in Cl^--sufficient and Cl^--depleted PS II particles (pH 7.5) with an initial distribution of $Q_B : Q_B^- = 50 : 50$. Samples were preilluminated (30 s) with continuous light, briefly relaxed (20 °C, 5 min) and then excited by one to six flashes (fl) at 20 °C. Left panel, Cl^--sufficient control in 20 mM NaCl; Right panel, Cl^--depleted by replacement with 70 mM Na_2SO_4.

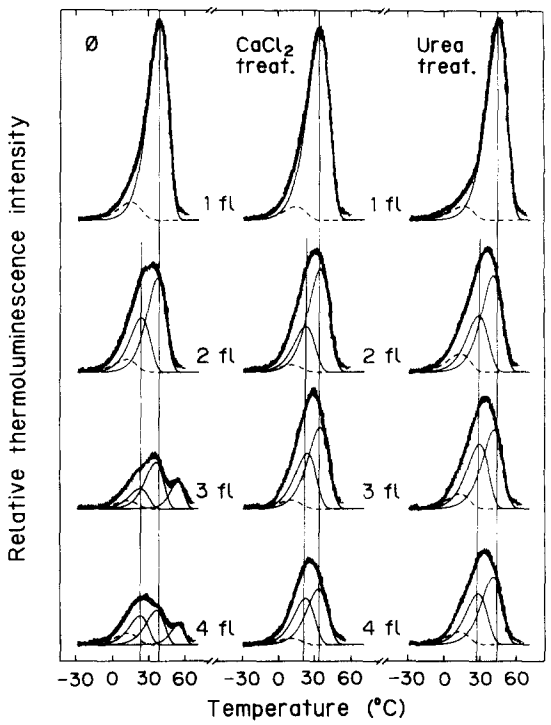

Fig. 5. Deconvolution of glow curves of 33 kDa extrinsic protein-removed PS II particles (pH 5.5). Dark-adapted particles were depleted of the three extrinsic proteins in complete darkness, and then excited by one to four flashes (fl) at 20 °C. Left, untreated control; middle, depleted by $CaCl_2$ treatment; right, depleted by urea/NaCl treatment. All samples contained 20 mM NaCl. Peak heights of the glow curves were normalized on the basis of the intensities after the first flash. Solid and broken thin curves are the components assumed by curve fitting.

$S_2Q_B^-$ (B_2 band) and $S_3Q_B^-$ (B_1 band) components, experiments were done at pH 5.5. The oscillations of the two components in untreated control particles were very similar to those shown in Fig. 3, except that the so-called C-band peaking at 55 °C was observed after the third or further flashes (left panel). The C-band can be observed only at low pH, and is proposed to be related to $(S_0 + S_1)$ [39].

As shown by the upper two curves in the middle and right panels of Fig. 5, the glow curves and their deconvoluted components in both the $CaCl_2$-washed and urea/NaCl-washed particles after the first and second flashes were very similar to those of the unwashed control. Notably, after the second flash, both washed samples showed a clear generation of the low-temperature component (B_1-band) arising from the $S_3Q_B^-$ charge pair at the expense of the high temperature component (B_2-band) arising from the $S_2Q_B^-$ charge pair. The two components thus generated after the second flash did not change any more on repeated excitation by the third and fourth flashes (lower two curves in the middle and right panels). These data are consistent with the observation by Ono and Inoue [25] and support their view that in the absence of the three extrinsic proteins (at low Cl^- concentration), the S-state transition can proceed to S_3, but not beyond S_3. However, it must be noted that the quantum yield of S_2–S_3 conversion by the third flash is far lower than that by the second flash. This can be seen clearly when the middle two pairs of glow curves (in the middle

and right panels) are compared: the second flash markedly induced the B_1-component ($S_3Q_B^-$) concomitant with the decrease in the B_2-component ($S_2Q_B^-$), but the third flash neither increased the B_1-component much nor decreased the B_2-component, although the relative height of B_1/B_2 was slightly increased. It thus appears that in the absence of the extrinsic proteins, the first two photoevents proceed normally at a high efficiency, but the following photoevents are blocked, even though some S_2 centers remain after the second flash.

Formation of the S_3 state in the absence of the extrinsic proteins was confirmed by another method. When the flash illuminated (at room temperature) PS II particles are cooled and further illuminated at $-196\,^\circ C$ with continuous light, one electron is transferred from cytochrome *b*-559 to Q_A [26]. Since the oxidized form of cytochrome *b*-559 does not participate in a thermoluminescence charge recombination, the low-temperature illumination results in the creation of one extra negative charge on the acceptor side [26]. On warming such double-illuminated samples, the extra negative charge on Q_A^- moves to Q_B at around $-30\,^\circ C$ [40]. However, when the centers are in S_3 state (after two-flash illumination at room temperature), the $S_3Q_A^-$ charge recombination occurs in some of the centers to emit the so-called A-band at around $-15\,^\circ C$. This process competes with the forward transport of the electron on Q_A^-, so that we see both $S_3Q_A^-$ component (A-band) and $S_3Q_B^-$ component (B_1-band) in two or more flash preilluminated samples. Detailed mechanisms for A-band emission are described in Ref. 40.

Fig. 6 shows the glow curves of urea/NaCl-washed samples measured under such excitation conditions. The control samples showed the A- and B_1-bands after two-flash preillumination, and both bands oscillated depending on the number of preflashes. The oscillation was of flash 2,6-maxima pattern (instead of flash 1,5-maxima pattern), even though the samples were of the thoroughly dark-adapted type. This is due to the apparent inversion of the $Q_B : Q_B^-$ ratio due to the one extra negative charge as mentioned above. (It has been confirmed that a flash 2,6-maxima pattern is observed when the $Q_B : Q_B^-$ ratio is lower than 50 : 50 (see, for example Refs. 26, 29, 38).) It is of note that these glow curves clearly demonstrate the

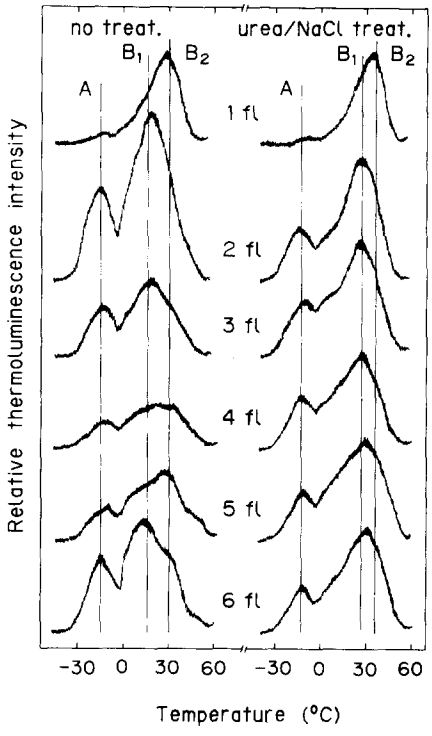

Fig. 6. Oscillation of thermoluminescence glow curves in 33 kDa protein depleted PS II particles (pH 5.5) measured by low temperature post illumination protocol. Dark-adapted particles were excited by one to six flashes (fl) at 20 °C followed by 1 min continuous illumination at −196 °C. This illumination protocol provides one extra electron on the acceptor side and eventually inverts the $Q_B : Q_B^-$ ratio (see text and refs. 26,35,38). Left, untreated control (no treat); right, urea/NaCl treatment. All samples contained 20 mM NaCl. Peak heights of the glow curves were normalized on the basis of the intensities after the first flash.

sequential transitions in S_1 centers, the major (72.5%) population in dark-adapted conditions: $S_2Q_B^-$ (B_2-band) is the dominant component after the first and fifth flashes, whereas $S_3Q_B^-$ and $S_3Q_A^-$ (B_1- and A-bands) are the dominant components after the second and sixth flashes. These results are well explained by the mechanism of thermoluminescence oscillation [26,29,38].

The glow curves of urea/NaCl-washed samples measured by the protocol described above, are shown in the right panel. After the first flash only the B_2-band ($S_2Q_B^-$ component) could be seen, but after the second flash, both the B_1- and A-bands

were clearly induced, indicative of formation of $S_3Q_B^-$ and $S_3Q_A^-$ components, respectively. The heights of these two bands were appreciably lower than those in control. This is due to the conversion of cytochrome *b*-559 from high-potential to low-potential form in part of the centers by removal of the extrinsic proteins [41], which limits the amount of extra negative charge on the acceptor side [40]. On increasing the flash number, the glow curve did not change any more, but kept the same shape and amplitude after the third and further flashes. This agrees with the results shown in Fig. 5 and indicates that the S-state transition in the absence of the extrinsic proteins is inhibited after two flashes and cannot proceed beyond S_3.

Fig. 7 summarizes the oscillation of the B-band (a composite of B_1- and B_2-bands) in Cl^--depleted and extrinsic protein-depleted PS II particles. In urea/NaCl-washed particles, the B-band oscillation was similarly interrupted after the second flash (solid circles) under the initial $Q_B : Q_B^-$ ratios of both 75 : 25 (panel A) and 25 : 75 (panel B), indicative of S_3 formation but interruption of further transitions. The interrupted oscillation was partially restored by reconstitution with 33 kDa extrinsic protein (half-solid circles), indicating that only the 33 kDa protein bears the essential role among the three extrinsic proteins.

In Cl^--depleted particles, the oscillation appeared to be interrupted after the second flash when the initial $Q_B : Q_B^-$ ratio was 75 : 25 (solid circles, panel C), but when the initial $Q_B : Q_B^-$ was 50 : 50 the interruption occurred after the first flash (solid circles, panel D). As discussed in Ref. 37 and also in this paper, this is probably due to the artificial acceptor side-effect which manifests under uneven initial distribution of $Q_B : Q_B^-$. Thus, it is concluded that Cl^- depletion blocks the S_2–S_3 transition. When Cl^- is readded to depleted PS II, the normal oscillation of the B-band is restored regardless of the initial $Q_B : Q_B^-$ ratio (half-solid circles, panels, C, D).

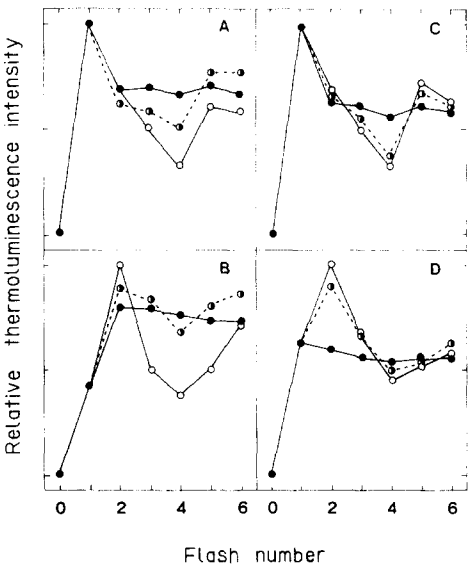

Fig. 7. Effects of Cl^- depletion or repletion and removal or reconstitution of the 33 kDa extrinsic protein on oscillation patterns of the B-band intensity. (A) Dark-adapted particles (pH 5.5, 20 mM NaCl) were excited by a series of flashes at 20 °C: untreated control (○); urea/NaCl-treated (●): reconstituted with 33 kDa protein (◑). (B) Dark-adapted samples (pH 5.5, 20 mM NaCl) were excited by series of flashes at 20 °C followed by 1 min continuous illumination at −196 °C. Symbols are the same as in (A). (C) Dark-adapted particles (pH 7.5) were excited by series of flashes at 20 °C; untreated control ○; Cl^--depleted by replacement with 70 mM Na_2SO_4 ●; Cl^--reconstituted by inclusion of 50 mM NaCl to the SO_4^{2-}-replaced sample (◑). (D) particles (pH 7.5) with initial distribution of $Q_B : Q_B^- = 50 : 50$ attained by 30 s illumination at 20 °C followed by dark-adaptation for 10 min. Symbols are the same as in (C).

Effect of high salt concentration in the absence of extrinsic proteins

A high concentration of Cl^- is known to reverse many of the inhibitory effects brought about by removal of the 33 kDa protein: partial restoration of O_2 evolution [18,19], and partial recovery of the multiline EPR signal accompanied by its period-four oscillation [24]. These observations are interpreted as indicating that the enhanced Cl^- demand due to removal of the extrinsic protein is reversed by a high Cl^- concentration. In order to obtain information about the relationship between Cl^- depletion and removal of the 33 kDa protein, we attempted to remove Cl^- in the absence of the extrinsic proteins.

Urea/NaCl-washed membranes were suspended in a low-Cl^- medium (2 mM Cl^-, pH 7.5), which are the conditions to facilitate Cl^- depletion by replacement with SO_4^{2-} in control PS II particles having extrinsic proteins. Since a very high concentration of Cl^- (100–200 mM) is

required to reverse the effect of removal of the 33 kDa protein [18,21,24], it was expected that the above Cl^- concentration (2 mM Cl^-) would include some Cl^--depleted characteristics of thermoluminescence. Contrary to this expectation, however, the peak positions of both the B- and Q-bands were practically unchanged (Fig. 8, solid curves, a, b), although their band heights were lowered to about one-third compared to those in 20 mM Cl^-. This suggests that Cl^- depletion in the absence of the extrinsic proteins affects thermoluminescence in a different way from that in the presence of extrinsic proteins.

When 100 mM SO_4^{2-} was included in the medium (pH 7.5), the heights of the B- and Q-bands were doubled, accompanied by a slight upshift of the peak positions by about 3 and 5 °C, respectively (solid curves c, d). When 100 mM Cl^- was further included, the peak positions of the two bands were shifted to lower temperatures by 3 and 6 °C, respectively, concomitant with a slight increase in band height (broken curves c, d). These changes are analogous in their trends to those observed in the presence of extrinsic proteins, but are far less pronounced, again indicating that Cl^- depletion and repletion in the absence of the extrinsic proteins affect thermoluminescence in quite a different way.

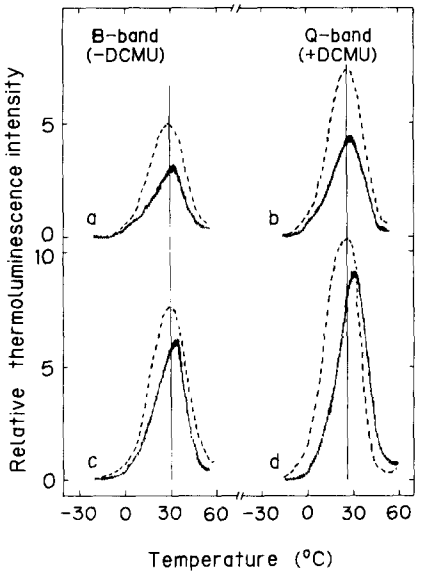

Fig. 8. Effect on thermoluminescence of Cl^- depletion by replacement with SO_4^{2-} in the absence of the 33 kDa extrinsic protein. Urea/NaCl-treated particles were suspended in 2 mM NaCl (pH 7.5) after two washes with the same buffer, and B- and Q-bands were excited by a single flash at 20 °C in the absence and presence of 10 μM DCMU (diuron), respectively. Salt additions are: (a), no addition (2 mM NaCl, solid), 100 mM NaCl (broken); (b), the same as in (a) except for 10 μM DCMU (≡diuron); (c), 100 mM Na_2SO_4 (solid), 100 mM Na_2SO_4 plus 100 mM NaCl (broken); (d), the same as in (c) except for 10 μM DCMU (diuron).

In a low Cl^- medium, the peak heights of the B- and Q-bands were significantly low, as mentioned above. This is partly due to the loss of Mn during washing with a low Cl^- medium, but when the same samples were measured in 100 mM Cl^-, both the B- and Q-bands were enhanced by factors of 1.6 and 1.7, respectively (broken curves a, b), indicative of good retention of Mn in the particles. The marked enhancement of thermoluminescence intensity by Cl^- seems to imply that in the absence of the extrinsic proteins, the yield of thermoluminescence depends more strongly on the ionic strength of the medium than on the presence of the extrinsic proteins.

Fig. 9 shows the glow curves of urea/NaCl-washed particles suspended with 200 mM NaCl (pH 5.5) and illuminated with a series of flashes. In the presence of a high concentration of Cl^-, the washed particles restored about 30% of the O_2 evolution (not shown). As shown in Figs. 2 and 5, urea/NaCl-washed particles in 20 mM NaCl show the B-band with the normal peak temperature of B_2-component after the first flash. In 200 mM NaCl, however, a broad band peaking at 42 °C was observed after the first flash (top curve, left panel). This band was somewhat similar to the SO_4^{2-}-induced band (not shown at pH 5.5, but shown at pH 7.5 in Fig. 8) and contained at least one additional high-temperature component other than the main normal B_2-band. The appearance of the high-temperature component may be due to the above-mentioned ionic strength effect. These results imply that the high concentration of Cl^- in the absence of the 33 kDa extrinsic protein induces an abnormal high-temperature band, which is in sharp contrast with the restoring functions of high Cl^- concentration in flash O_2 yield [23] and EPR measurements [24].

When illuminated with two or more flashes, the glow curves showed some oscillatory behavior; the marked change in total luminescence after the 2nd

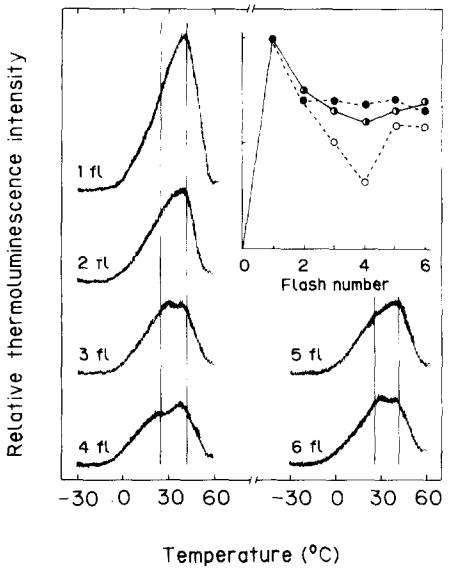

Fig. 9. Oscillation of glow curves of 33 kDa protein-depleted PS II particles suspended with high concentration of Cl^-. Dark-adapted particles were depleted of all the extrinsic proteins by urea/NaCl wash in complete darkness and suspended with 200 mM NaCl at pH 5.5. Thermoluminescence was excited by a series of flashes (fl) at 20 °C. The solid-line oscillation pattern in the inset (◑) was obtained from the area under the glow curves. Broken-line patterns with symbols (○ and ●) are those of untreated and depleted particles (in 20 mM Cl^-), respectively, reproduced from Fig. 7A.

flash was followed by appreciable changes in the shape of glow curves. The appearance of shoulders at 30 °C and 28 °C after the third and fourth flashes, respectively, may be correlated to the formation of B_1-band ($S_3Q_B^-$) as we observed in 20 mM Cl^- (Fig. 5). It was difficult to observe a period-four oscillation based on the peak height of the thermoluminescence band(s), but when the total luminescence (area under the glow curve) was plotted against the flash number, the pattern shown in the margin of Fig. 9 was obtained (half-solid circles). The amplitude of oscillation was appreciable when compared with the interrupted pattern in 20 mM Cl^- (solid circles), but was very faint when compared with that in normal particles (open circles).

The very faint oscillatory behavior in thermoluminescence is consistent with the strongly damped oscillation of the flash O_2 yield reported in Ref. 34, but contrasts to the clear period-four oscillations reported for the flash O_2 yield [23] and the multiline EPR signal [24] of 33 kDa protein-depleted particles suspended in 200 mM Cl^-. This is presumably because the flash O_2 yield and EPR measurements in Ref. 23,24 selectively detected the restored active centers (by ignoring the dead centers), whereas the thermoluminescence nonselectively detected both the dead and the active centers, as demonstrated by the abnormal thermoluminescence band in the centers inactivated by Cl^- depletion (Fig. 1).

Concluding remarks

The present study demonstrates that Cl^- depletion and removal of the 33 kDa extrinsic protein modify the charge stabilization conditions in PS II in different ways: the former affects both $S_2Q_B^-$ and $S_2Q_A^-$ charge pairs, and thereby interrupts the S-state turnover at S_2, whereas the latter specifically affects $S_2Q_B^-$, and thereby interrupts the S-state turnover at S_3 (at a low Cl^- concentration of 20 mM). These results clearly indicate that removal of the extrinsic protein affects O_2 evolution not by enhancing the demand for Cl^-, but by some other mechanism. We consider that the role of the extrinsic protein is not merely to mitigate the Cl^- demand, but is to facilitate the S-state turnover by providing a structural integrity required particularly for S_3–(S_4)–S_0 transition, even though many of the inhibitory effects due to the protein removal are appreciably reversed by a high Cl^- concentration. Probably, the high concentration Cl^- effect to partially restore O_2 evolution in the absence of the extrinsic protein is largely different in its mechanism from the low-concentration Cl^- effect to completely restore the inhibition of S_2–S_3 transition, the high-affinity and the low-affinity Cl^- effect as suggested by Damoder et al. [12]. This view may be consistent with the observation that the abnormal properties of the $S_2Q_A^-$ pair induced by removal of the protein cannot be reversed by a high Cl^- concentration but can be by rebinding of the extrinsic protein, as shown in this study and in Ref. 31.

As to the $S_2Q_A^-$-specific effect of removal of the 33 kDa protein, we provide a very tentative interpretation, a fortuitous cancellation of the effects on S_2 and Q_B^-. In view of the recently proposed

structural analogy between the higher plant PS II and the bacterial reaction center [42,43], in which Q_B and Q_A are assumed on D1 and D2 proteins, respectively, the preferential effect on Q_B^- of the protein removal with less influence on Q_A^- seems to suggest a closer association of this extrinsic protein with D1 than D2. This hypothesis would be an interesting facet to be persued in future studies.

Acknowledgements

This work was supported by a Grant on Solar Energy Conversion by Means of Photosynthesis awarded to The Institute of Physical and Chemical Research (RIKEN) by The Science and Technology Agency of Japan, and partly by a Grant-in-Aid No. 60304093 given by The Ministry of Education, Science and Culture of Japan.

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