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Removal of 33 kDa extrinsic protein specifically stabilizes the $S_2Q_A^-$ charge pair in photosystem II

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Removal of the 33 kDa extrinsic protein from photosystem (PS) II membranes resulted in markedly increased stabilization of the $S_2Q_A^-$ charge pair as measured by thermoluminescence. The stabilization increase was specific for the $S_2Q_A^-$ charge pair and did not require any special herbicide. The effect was fully reversed by reconstitution with the 33 kDa protein, but not at all by high concentrations of Cl^-, as opposed to those effects known to be reversed by 200 mM Cl^-. The data are interpreted as indicating a structural change of the donor and/or acceptor side of PS II dependent on association with the 33 kDa extrinsic protein.

33 kDa protein; Photosystem II; S state; Thermoluminescence; Oxygen evolution

1. INTRODUCTION

Three extrinsic proteins with approximate molecular masses of 16, 23 and 33 kDa are known to be involved in photosynthetic oxygen evolution by higher plant PS II [1,2]. Among these, the 16 and 23 kDa proteins may be less essential because the partial inhibition of oxygen evolution in their absence [3,4] can be fully restored by Cl^- and/or Ca^{2+} [5,6], and also because these proteins are missing in cyanobacterial oxygen-evolving system [7,8]. The crucial role of the 33 kDa protein in PS II donor side reactions is shown by complete inhibition of oxygen evolution after Mn-preserving removal of all the three extrinsic proteins [9,10]. The inhibition is reversed by rebinding of extracted 33 kDa protein [11,15]. Although the S_2 state multiline EPR signal cannot be detected in the absence of the 33 kDa protein at low (<20 mM) Cl^- concentrations [13,14], storage of two equivalents of positive charges in the water-oxidizing enzyme has been proposed based on thermoluminescence measurements. This observation is interpreted as suggesting a role of the protein in ensuring the normal S_3 to S_4 transition [16]. However, recent findings that the 33 kDa protein-depleted membranes partially restore the activity of oxygen evolution [12,15,17], being accompanied by oscillation of the S_2 state EPR multiline signal [18] in the presence of a high concentration (200 mM) of NaCl, may appear to suggest that the removal of the protein simply enhances the demand for Cl^- [17] without any major damage in the Mn cluster of the water-oxidizing enzyme [18,19]. The 200 mM Cl^--restored oxygen evolution, however, has some abnormal characteristics such as low activity [12,15,17], high miss factor [20] and increased stability of S_2 and S_3 states [21]. These results seem to suggest that the 33 kDa protein has some specific functions in PS II electron

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Abbreviations: Chl, chlorophyll; DCMU, 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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transport which cannot be replaced by high concentrations of Cl^-.

By means of thermoluminescence we recently found a substantial stabilization of the $S_2Q_A^-$ charge pair upon removal of the three extrinsic proteins [22]. Here we report some unique properties of this stabilization effect, e.g. $S_2Q_A^-$ specificity and high Cl^- irreversibility.

2. MATERIALS AND METHODS

Oxygen-evolving PS II membranes were prepared from spinach thylakoids as described [16]. Before measurements or further treatments, the membranes were washed twice with 400 mM sucrose/20 mM NaCl/40 mM Mes-NaOH (pH 6.5) and then dark adapted for 5 h at 0°C to ensure complete relaxation of both the donor and acceptor sides of PS II.

Removal of the 16 and 23 kDa extrinsic proteins was attained by 1.5 M NaCl treatment as described in [20]. Mn-preserving removal of all the three 16, 23 and 33 kDa extrinsic proteins was attained by either 1.5 M $CaCl_2$ treatment [9] or urea/NaCl treatment [10]. The treated samples were washed twice with 400 mM sucrose/200 mM NaCl/4 mM Mes-NaOH (pH 6.5) and then resuspended in the same medium. All treatments were done at 0°C under dim green safe light.

The 33 kDa extrinsic protein was extracted with 20 mM alkaline Tris from NaCl-washed PS II membranes, concentrated by ultrafiltration with an Amicon PM 10 Diaflo membrane. For reconstitution, various amounts of the extracted protein were added to the urea/NaCl-washed membranes as in [11]. Polypeptide compositions of the membranes were checked by SDS-PAGE as usual [9]. The amount of bound 33 kDa protein was determined by densitometry of the stained gels using the 43 and 47 kDa proteins as inner standard.

Thermoluminescence was measured with dark-adapted samples diluted with 400 mM sucrose/40 mM Mes-NaOH (pH 5.5) and supplemented with the necessary amount of NaCl to reach 20 or 200 mM Cl^- and 300 μg/ml Chl. After excitation with a 5 μs xenon flash(es) at saturating intensity, thermoluminescence glow curves were recorded as in [23].

3. RESULTS

The thermoluminescence after one flash excitation of dark relaxed PS II membranes originates from recombination of the $S_2Q_B^-$ charge pair (B-band) in the absence, or of the $S_2Q_A^-$ charge pair (Q-band) in the presence of an inhibitor of electron transfer between Q_A and Q_B (e.g. DCMU) [23,24]. Based on the theoretical description of thermoluminescence events in the photosynthetic apparatus [25,26], the peak position of a glow curve approximately reflects the stabilization energy of the recombining charges: a higher peak position implies a larger stabilization energy and in turn a smaller redox span between the separated charges.

As fig.1 shows, removal of the 16 and 23 kDa extrinsic proteins by NaCl washing practically did not affect the glow curve (curves a,b). In contrast, when all the 16, 23 and 33 kDa extrinsic proteins were removed by $CaCl_2$ washing, the peak temperature of the Q-band was elevated by about 20°C (curve c, right) as compared with the control sample, although the peak temperature of the B-band was lowered by about 5°C (curve c, left). Essentially the same effects were obtained when urea/NaCl washing was used to remove the three extrinsic proteins (curve d, left and right). Notably, the inclusion of 200 mM NaCl in the medium did not reverse the elevated peak temperature of the Q-band, but resulted in the appearance of a shoulder at around 40–45°C due to a component with even higher emission temperature (curve e, right). This high-temperature component can also be seen for the B-band under high-Cl^- conditions as an upward shift of the peak temperature by about 5°C (curve e, left).

The similar peak temperatures of the B- and Q-bands in $CaCl_2$- or urea/NaCl-washed samples may lead to the idea that these treatments have damaged the DCMU-binding site. However, this possibility was excluded by the experiment shown in fig.2. In the presence of DCMU, the Q-band (emitting at 34°C) of the washed sample was fully charged by the 1st flash and its amplitude did not change on further flash excitation (curve a). In the absence of DCMU, on the other hand, the B-band (at 38–40°C) showed oscillatory behavior during the 1st and 2nd flashes and stopped oscillation afterwards (curve b). The interruption of oscilla-

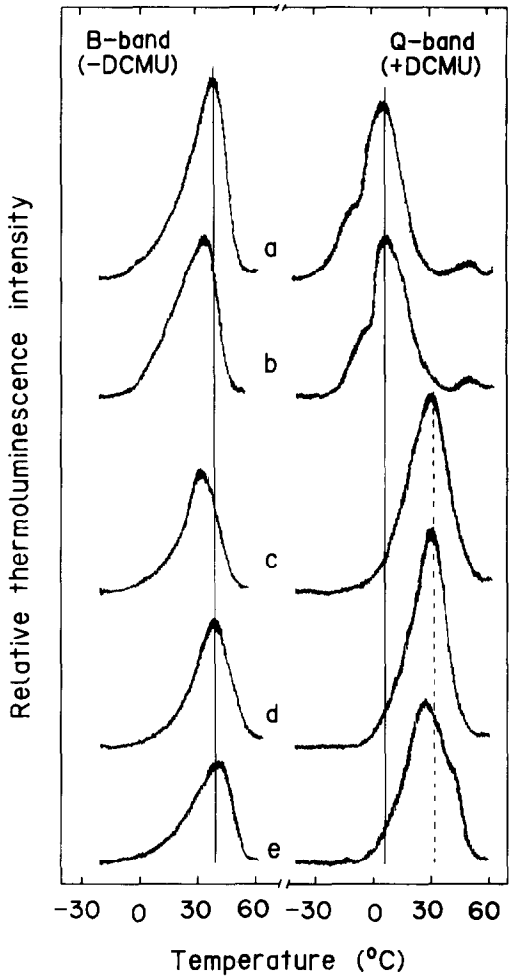

Fig.1. Effect of removal of extrinsic proteins on thermoluminescence glow curves of PS II membranes. B-bands (left) and Q-bands (right) were excited by a single flash at 20 and −5°C, in the absence and presence of 10 μM DCMU, respectively. (a) Untreated control, (b) 1.5 M NaCl wash (16 and 23 kDa proteins removed), (c) 1.5 M $CaCl_2$ wash (16, 23 and 33 kDa proteins removed), (d) 2.7 M urea/0.2 M NaCl wash (16, 23 and 33 kDa proteins removed), (e) the sample for (d) was supplemented with 200 mM NaCl. With the exception of (e) all samples contained 20 mM NaCl. The distortion of glow curves at around 0°C is due to the solid-liquid phase transition of the samples.

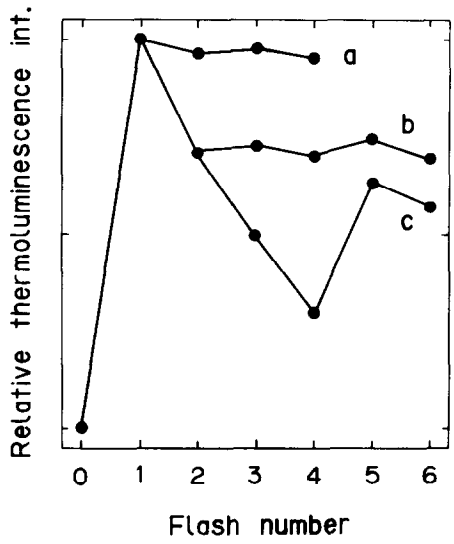

Fig.2. Oscillation of thermoluminescence intensity of PS II membranes under single-turnover flashes. The three extrinsic proteins (16, 23 and 33 kDa) were removed by 2.7 M urea/0.2 M NaCl washing, and the washed membranes were suspended in low (20 mM) Cl^- medium. (a) Q-band (34°C) of washed membranes in the presence of 10 μM DCMU, (b) B-band (40°C) of washed membranes, (c) B-band (40°C) of unwashed control. Thermoluminescence was excited at 20°C, and the peak heights were normalized on the basis of the heights after the 1st flash.

tion after the 2nd flash has been interpreted as the inhibition of the S_3 to S_0 transition in the absence of the 33 kDa protein (at low Cl^- concentration) [16]. The fact that DCMU thus converts the oscillation pattern from curve b to curve a confirms that the herbicide-binding site is retained in the washed membranes, and that the $S_2Q_A^-$ charge pair is specifically stabilized in the absence of the 33 kDa extrinsic protein.

The enhancement of $S_2Q_A^-$ stabilization by removal of the extrinsic proteins was examined with different types of herbicides. As fig.3 shows, both the ioxynil-induced Q-band and atrazine-induced Q-band were shifted to higher temperatures by about 25°C on removal of the 33 kDa protein (curves a,b, left and right). The extent of the upward shift was more or less the same as in the case of the DCMU-induced Q-band. The ioxynil-induced Q-band and atrazine-induced Q-band in untreated membranes usually appear at lower temperatures than the DCMU-induced Q-band by 20 and 4°C, respectively [27], and these relationships are largely preserved after removal of the 33 kDa protein. Chemical reduction of Q_B by 5 mM Na dithionite is known to convert the B-

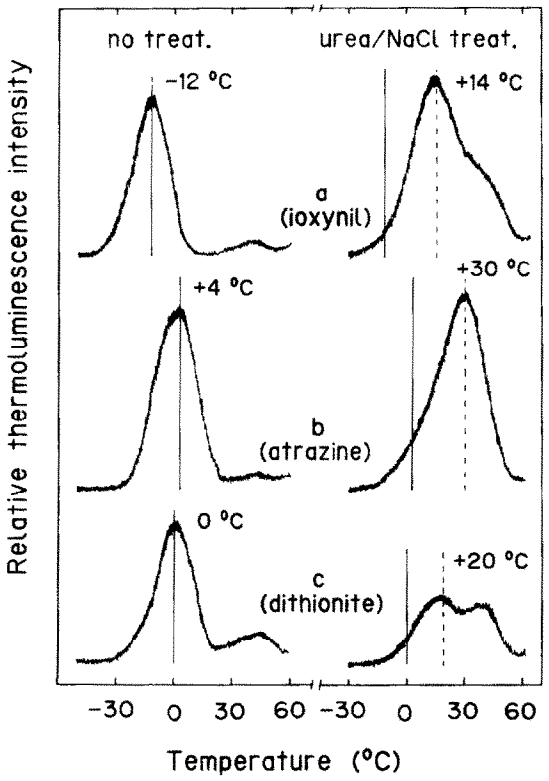

Fig.3. Effect of removal of the three (16, 23 and 33 kDa) extrinsic proteins on the peak position of the Q-bands induced by different treatments. The extrinsic proteins were removed by 2.7 M urea/0.2 M NaCl washing. All samples were suspended in a low Cl^- medium (20 mM NaCl), and 30% glycerol was added to avoid the distortion of glow curves at around 0°C. Thermoluminescence was excited by a single flash illumination at −20°C. Left glow curves, normal PS II membranes; right glow curves, washed PS II membranes depleted of the three extrinsic proteins. Q-bands were induced by (a) 30 μM ioxynil, (b) 15 μM atrazine and (c) 5 mM dithionite (0.5 mM in washed membranes).

band to Q-band in untreated membranes [23]. The chemically induced Q-band was also shifted to a higher temperature by about 20°C on removal of the 33 kDa protein (curves c, left and right). The glow curve (c, right) shows both the Q- and B-bands. This is due to the lower concentration of dithionite (0.5 mM): at higher concentrations, thermoluminescence is totally abolished, probably because the Mn atoms are so exposed to dithionite in the absence of the three extrinsic proteins that they easily lose positive charges by chemical reduction. Similar effects of exogenous reducing agents have been reported for NaCl-washed membranes [28].

The fact that the $S_2Q_A^-$ stabilization effect can be commonly observed with various types of herbicides suggests that the effect is not due to a change in the mode of herbicide binding, but is due directly to a modification of the $S_2Q_A^-$ properties induced by removal of the proteins. The strict dependency on the 33 kDa extrinsic protein was confirmed by the reconstitution experiments shown in fig.4A. As shown by glow curves a and

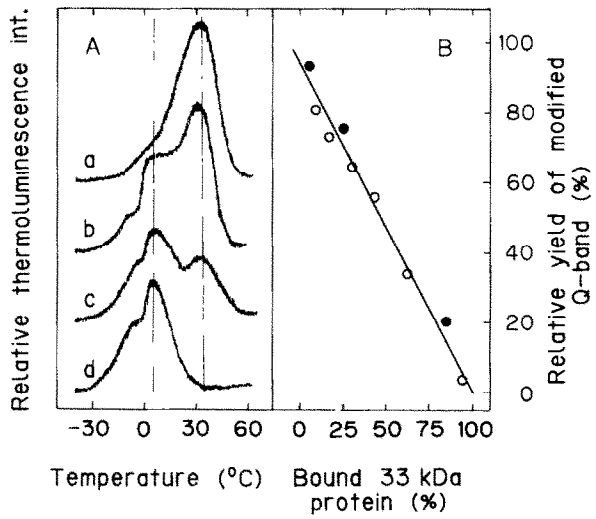

Fig.4. Effect of reconstitution with 33 kDa extrinsic protein on the peak position of the Q-band. Urea/NaCl-washed membranes were reconstituted with various amounts of the extrinsic protein, and suspended in a low Cl^- medium (20 mM NaCl). Thermoluminescence was excited by a single flash at −20°C. (A) Glow curves of the PS II membranes, (a) no reconstitution; (b,c) partial reconstitution with a limited amount of 33 kDa protein; (d) full reconstitution with excess amount of 33 kDa protein. (B) Relationship between the relative yield of the modified Q-band (34°C) and the relative amount of the 33 kDa protein associated with the membranes: open and solid circles denote data obtained by partial reconstitution and partial depletion, respectively. Relative yield of the modified Q-band was determined by curve fitting by assuming the glow curves are composed of normal and modified Q-bands. High emission intensity of the modified Q-band (by a factor of about 1.7) was taken into account.

d, the elevated peak temperature of the Q-band arising from the modified $S_2Q_A^-$ pair was almost completely reversed to the normal peak temperature by reconstitution with the 33 kDa protein. Interestingly, when the amount of 33 kDa protein was limited, the glow curves showed both the modified and normal (reversed) Q-bands simultaneously (curves b,c). This implies a complete restoration in a limited subpopulation of the centers, i.e. an all-or-none type reconstitution of normal centers by rebinding of the 33 kDa protein. In fact, there was a linear relationship between the relative amount of 33 kDa protein rebound and the relative amplitude of the modified Q-band (with higher peak position) (fig.4B), and the same relationship can be obtained by either partial reconstitution (open circles) or partial depletion (solid circles) of the 33 kDa protein. However, the maximal (95%) reconstitution of the normal Q-band was accompanied by only about 60% of oxygen evolution. This may imply that the structural integrity required for charge stabilization of $S_2Q_A^-$ charge pair is not as strict as required for oxygen evolution.

4. DISCUSSION

It is shown in this study that the removal of 33 kDa extrinsic protein selectively affects the stabilization of the $S_2Q_A^-$ charge pair with practically no effect on the $S_2Q_B^-$ charge pair. This selective effect is in sharp contrast with the effect of Cl^- depletion: in Cl^--free PS II, the redox spans of both $S_2Q_B^-$ and $S_2Q_A^-$ charge pairs are affected in the same direction and to the same extent, being decreased by about 60–80 mV [22], and this phenomenon is interpreted as being due to a specific modification of the redox properties of S_2, the common counterpart of both charge pairs, with no or very slight influence on Q_A^- and Q_B^-. Since a shift in thermoluminescence peak temperature can similarly result from the influences on either the donor (positive) or acceptor (negative) sides [22], the following alternatives must be taken into account in interpreting the selective influence on $S_2Q_A^-$. Removal of the 33 kDa protein (i) specifically modifies the redox properties of Q_A^- with no influence on those of Q_B^- and S_2, or (ii) modifies the redox properties of both Q_B^- and S_2 with no or less influence on those of Q_A^-, provided the changes in redox potential of Q_B^- and S_2 approximately compensate with each other to result in an almost unchanged redox span of $S_2Q_B^-$. Of these two interpretations, the former interpretation (i) does not seem likely in view of the well established role of the 33 kDa extrinsic protein in stabilizing Mn binding on the donor side of PS II [10,12,16]. The latter interpretation (ii) appears more likely because it assumes a modification of S_2 properties on the donor side, and this assumption is relevant to reports [13,14] that the multiline signal is not detected in the absence of the 33 kDa extrinsic protein at low Cl^- concentration. It appears difficult to assume that the removal of the extrinsic protein induces a structural change on the acceptor side. However, in view of the report that the affinity to various artificial electron acceptors changes after $CaCl_2$ washing [20], it may not be irrational to assume an effect on the properties of Q_B^-. The effect must be specific for Q_B^- to account for cancellation of the effect for the $S_2Q_B^-$ pair but the retention of the effect for the $S_2Q_A^-$ pair. The different effects on the two quinone acceptors are due to the differences in their environment: Q_A is buried more deeply than Q_B [29], thus being more resistant to the high concentration salt treatment.

From the present data, we tentatively conclude that the removal of the 33 kDa protein selectively affects the properties of the $S_2Q_A^-$ charge pair. However, in spite of the ambiguities as to the mechanism of its selective effect, the effect is of unique characteristics among the other effects caused by removal of the 33 kDa extrinsic protein, since it cannot be reversed by 200 mM Cl^- as opposed to the EPR multiline signal or oxygen evolution, but can only be specifically reversed by reconstitution with the 33 kDa extrinsic protein.

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