Structural and Functional Modulation of the Manganese Cluster in Ca^{2+}-Depleted Photosystem II Induced by Binding of the 24-Kilodalton Extrinsic Protein†

Taka-aki Ono,* Seikichi Izawa,‡ and Yorinao Inoue

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

Received January 30, 1992; Revised Manuscript Received May 14, 1992

ABSTRACT: Depletion of functional Ca^{2+} from photosystem (PS) II membranes impairs O_2 evolution. Redox properties of the Mn cluster as probed by thermoluminescence were modified differently in Ca^{2+}-depleted PSII depending on the procedure for Ca^{2+} extraction. Ca^{2+} depletion by low-pH treatment gave rise to an abnormally modified S_2 state exhibiting a thermoluminescence band with elevated peak temperature accompanied by a marked upshift in threshold temperature for its formation, whereas Ca^{2+} depletion by NaCl washing in the light followed by the addition of EDTA could generate a similarly modified S_2 state only when the Ca^{2+}-depleted PSII was reconstituted with the 24-kDa extrinsic proteins. These results indicated that manifestation of the abnormal properties of the Ca^{2+}-depleted S_2 state is significantly contributed by the association of the 24-kDa extrinsic protein to PSII. It was inferred that the 24-kDa extrinsic protein regulates the structure and function of the Mn cluster in the absence of functional Ca^{2+} through a conformational modulation of the intrinsic protein(s) that bind(s) both Mn and Ca. Features of the extrinsic protein-dependent modulation of the Mn cluster were discussed in relation to the function of Ca^{2+} in O_2 evolution.

Photosynthetic water oxidation by photosystem (PS)[1] occurs at the O_2-evolving center, in which a tetranuclear Mn cluster is thought to provide the catalytic site for oxidation of water as well as charge accumulation [for reviews, see Brudvig et al. (1989) and Babcock et al. (1989)]. Four turnovers of the O_2-evolving center through five distinct oxidation states labeled S_0–S_4 are required for production of one molecule of oxygen from two molecules of water (Joliot & Kok, 1975). The minimum PSII complex capable of O_2-evolving consists of seven major intrinsic proteins and one extrinsic protein (33-kDa protein) (Ikeuchi & Inoue, 1986). In PSII membranes, a more native PSII complex, two more extrinsic proteins (16- and 24-kDa proteins) are associated with the O_2-evolving center. The two extrinsic proteins have been proposed to facilitate the retention of Cl^- and Ca^{2+} in the center, respectively (Ghanotakis et al., 1984a,b; Miyao & Murata, 1984; Akabori et al., 1984), the cofactors indispensable for normal operation of the O_2-evolving device, and a specific turnover step of S-state cycling is interrupted when these cofactors are depleted.

In higher plants, it has been shown that two atoms of Ca at the minimum are associated with an O_2-evolving PSII complex with different affinity (Cammarata & Cheniae, 1987; Ono & Inoue, 1988; Shen et al., 1988; Kalosaka et al., 1990) and one of the two is released by low-pH treatment in the dark (Ono & Inoue, 1988). Treatment with high concentrations of NaCl in the light also removes Ca atom(s), but in this case, the 16- and 24-kDa extrinsic proteins are concomitantly released (Cammarata & Cheniae, 1987; Kalosaka et al., 1990). After these treatments, O_2 evolution is drastically inhibited, but addition of exogenous Ca^{2+} restores the activity to a large extent. In low-pH-treated membranes, a long incubation (up to 40 min) with exogenous Ca^{2+} is needed to restore the activity to be resistant to exogenous chelators. A similar phenomenon has been observed when NaCl-washed PSII membranes are reconstituted with the 16- and 24-kDa proteins (Ghanotakis et al., 1984a). These results are interpreted to mean that binding of the 24-kDa protein constitutes a barrier that prevents the Ca^{2+}-binding site in PSII from rapid equilibration with exogenous Ca^{2+}. When the 16- and 24-kDa proteins are removed by NaCl washing in the dark, the resulting PSII retains most of the functional Ca^{2+}, and light illumination is needed to make the retained Ca^{2+} liberate (Dekker et al., 1984; Ono & Inoue, 1986; Miyao & Murata, 1986; Boussac & Rutherford, 1988a). As these results suggest, the 24-kDa protein is involved in regulation of the stability of Ca^{2+} in its site of action.

There is general agreement that Ca^{2+} depletion blocks the normal cycling of the S state, but its exact interruption step remains a matter of debate despite intensive efforts to reconcile the contradictory results from different laboratories [for a review, see Yocum (1991)]. Among those divergent results, a dark-stable S_2 state having a long life has been observed by several laboratories: after stringent Ca^{2+} depletion by NaCl washing in the light plus EGTA followed by dialysis in the light to let the 16- and 24-kDa proteins rebind to the membrane (Boussac et al., 1989); after NaCl washing in the light plus EDTA (Ono & Inoue, 1990b); after low-pH treatment in the dark (Sivaraja et al., 1989; Ono & Inoue, 1989a,b). The dark-stable S_2 state is characterized by its altered multiline EPR signal with reduced hyperfine splitting and thermoluminescence (TL) band with elevated peak temperature. In NaCl-washed PSII, the dark-stable S_2 state is found in the treated sample as the S_2 state has been generated by light

† This work was supported by grants for the Photosynthetic Sciences and Biodesign Research Program (to T.O.) at The Institute of Physical and Chemical Research (RIKEN) given by the Science and Technology Agency (STA) of Japan and in part by MESC Grant-in-Aid for Cooperative Research 01300009. S.I. was a recipient at RIKEN supported by the U.S.–Japan Program for Cooperation on Photoconversion and Photosynthesis.

* To whom correspondence should be addressed.

‡ Present address: Department of Biological Sciences, Wayne State University, Detroit, MI 48202.

[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PS, photosystem; Q_A, primary quinone acceptor of photosystem II; TL, thermoluminescence.

0006-2960/92/0431-7648$03.00/0

illumination during the NaCl washing, whereas in low-pH-treated PSII, its generation requires a further illumination to induce the S_2 state. Upon addition of exogenous Ca^{2+}, both two dark-stable S_2 states are converted to normal S_2 in the dark and start decaying rapidly (Ono & Inoue, 1989a; Ono et al., 1991).

In low-pH-treated membranes, Ca^{2+} depletion induces a dramatic change in the temperature dependence of the S_1 to S_2 transition. The half-inhibition temperature for induction of the modified multiline S_2 signal or the high-temperature-peaked TL band is markedly upshifted by over 90 °C, and this upshift is largely reversed by addition of exogenous Ca^{2+} (Ono & Inoue, 1990a; Ono et al., 1991). This phenomenon has been confirmed later to occur also in NaCl-plus-chelator-washed PSII reconstituted with the 16- and 24-kDa proteins (Boussac et al., 1990b). On the basis of the observation that the Mn K-edge energy value of the XANES (X-ray absorption near-edge structure) spectrum reversibly shifts to a lower energy after low-pH treatment (Ono et al., 1991), it is assumed that the Ca^{2+} depletion by low-pH treatment gives rise to a structural modulation of the Mn cluster.

In the present study, we investigated the effects of binding of the 24-kDa extrinsic protein on the redox properties of the Mn cluster by means of TL. The results show that the redox properties of the Mn cluster in Ca^{2+}-depleted PSII are directly modulated upon binding the 24-kDa protein, suggesting that the 24-kDa extrinsic protein is a sort of formative device that regulates the functional structure of the Mn cluster in concert with Ca^{2+}.

MATERIALS AND METHODS

Triton X-100 solubilized PSII membranes capable of O_2 evolution were prepared from spinach according to Berthold et al. (1981) with a modification (Ono & Inoue, 1986) and stored in liquid N_2 until use. After being thawed, the membranes were washed twice with 400 mM sucrose/20 mM NaCl/40 mM MES–NaOH (pH 6.5), suspended in the same medium, and dark-adapted for 4 h at 0 °C. All the following procedures were done under dim-green safe light or in complete darkness unless otherwise noted.

For NaCl washing, the membranes were resuspended in 2 M NaCl/400 mM sucrose/2 mM EDTA·2Na/40 mM MES–NaOH (pH 6.5) at a sample concentration of 0.5 mg of Chl/mL and incubated at 0 °C for 30 min. The membranes were then washed and resuspended in 400 mM sucrose/20 mM NaCl/0.5 mM EDTA·2Na/40 mM MES–NaOH (pH 6.5) (buffer A). The resulted membranes were denoted as NaCl/dark-washed membranes in this paper. Alternatively, the membranes were resuspended in 2 M NaCl/400 mM sucrose/40 mM MES–NaOH (pH 6.5) at a sample concentration of 0.5 mg of Chl/mL and incubated at 0 °C for 30 min under room light. EDTA·2Na was then added to the washed membranes at a final concentration of 1 mM, followed by further 5-min incubation in the dark. The resulted membranes (denoted as NaCl/light/EDTA-washed membranes) were washed once and resuspended in buffer A. For low-pH treatment, the membranes were suspended with 400 mM sucrose/20 mM NaCl/10 mM citrate–NaOH (pH 3.0) at 0 °C for 5 min, added with 0.1 volume of 400 mM sucrose/20 mM NaCl/500 mM MOPS-NaOH (pH 7.5), and then incubated for 10 min to facilitate the rebinding of extrinsic proteins. The resulted membranes (denoted as low-pH-treated membranes) were washed and resuspended in buffer A.

The 16- and 24-kDa extrinsic proteins were extracted as a crude mixture by washing the PSII membranes with 2 M NaCl/20 mM MES–NaOH (pH 6.5). The mixture was concentrated with an Amicon PM10 Diaflo membrane followed by repeated dialysis for 1 h against 10 mM NaCl/0.5 mM EDTA·2Na/10 mM MES–NaOH (pH 6.5). The total period of dialysis was as short as 4 h in order to minimize proteolytic breakdown. The 24-kDa protein was purified from this extract by column chromatography with Mono Q (Pharmacia) and dialyzed against 10 mM NaCl/0.5 mM EDTA·2Na/10 mM MES–NaOH (pH 6.5). For protein reconstitution, the PSII samples depleted of both 16- and 24-kDa proteins were incubated with the crude extract or purified protein at 0 °C for 20 min, washed twice with buffer A, and then resuspended in the same medium. Protein composition was analyzed by SDS–polyacrylamide gel electrophoresis as described in Ono et al. (1986). The gel was stained with Coomassie brilliant blue R-250. The densitogram of the stained gel was obtained with a dual-wavelength chromatoscanner (Shimadzu, CS-900), and the relative abundance of proteins was estimated from the peak area of the densitogram.

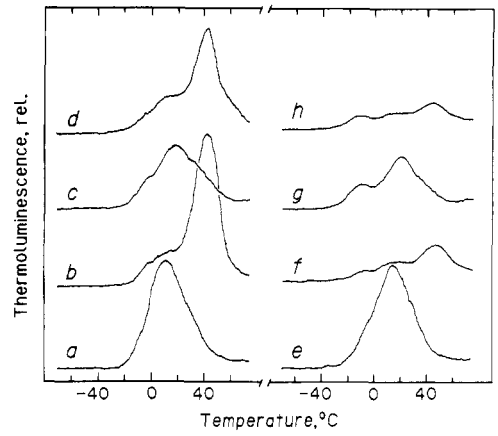

FIGURE 1: TL glow curves induced by single-flash excitation at 0 °C (left side) and at −40 °C (right side) of PSII membranes in the presence of 10 μM DCMU. Untreated control PSII (a, e); low-pH-treated PSII (b, f); low-pH-treated PSII followed by NaCl/dark washing (c, g); low-pH-treated PSII followed by NaCl/dark washing and then reconstituted with 16- and 24-kDa extrinsic proteins (d, h). Saturating amounts of the two extrinsic proteins were added for protein reconstitution.

For TL measurements, samples were excited with a saturating white Xe flash or a continuous red light (>630 nm) and cooled quickly in a liquid-N_2 bath, and then light emission during warming was recorded against the sample temperature as described (Ono & Inoue, 1990a). O_2-evolving activity was measured at 25 °C with a Clark-type O_2 electrode with phenyl-*p*-benzoquinone (1 mM) as electron acceptor as previously described (Ono & Inoue, 1988). DCMU (0.01 mM) was routinely added in TL measurements in order to ensure a single turnover of PSII. DCMU and phenyl-*p*-benzoquinone were dissolved in dimethyl sulfoxide as stock solutions.

RESULTS

Figure 1 shows the effects on TL glow curves of NaCl/dark washing and NaCl/dark washing followed by rebinding of the 16- and 24-kDa extrinsic proteins as compared with those of low-pH treatment. Upon excitation by a single flash at 0 °C in the presence of DCMU (left side), untreated control PSII showed a TL band peaking at 13 °C arising from charge recombination between S_2 and Q_A^- (curve a). In low-pH-treated PSII, however, the TL peak temperature from the same charge pair was markedly elevated to 43 °C (curve b).

As reported previously (Ono & Inoue, 1989a, 1990a), this upward shift of TL peak temperature results from modification of the S_2 state by Ca^{2+} depletion. When the low-pH-treated PSII was further washed with 2 M NaCl in darkness, the resulting PSII (denoted as low-pH NaCl/dark-treated PSII) showed a TL band peaking at 16 °C (curve c), more or less the same peak temperature as found for untreated control PSII. It appears that NaCl/dark washing cancels the effect caused by low-pH treatment as far as the TL properties are concerned. This cannot be due to a fortuitous restoration by contaminating Ca^{2+}, since EDTA was included in the medium throughout the treatment procedures. The low-pH-treated PSII is depleted of functional Ca^{2+} but retains the three (16, 24, and 33 kDa) extrinsic proteins that are associated with the O_2-evolving catalyst (Ono & Inoue, 1988). Since 2 M NaCl washing of PSII is known to selectively remove the 16- and 24-kDa extrinsic proteins (Kuwabara & Murata, 1983), the restoration of an almost normal TL band after NaCl/dark washing would be due to the removal of these two proteins from the low-pH-treated PSII. This interpretation is verified by the observation that rebinding of the two extrinsic proteins to low-pH NaCl/dark-treated PSII led the TL band again to exhibit an upshifted peak temperature (curve d).

The right side of Figure 1 shows the TL glow curves of the same samples after excitation by a single flash at –40 °C. The TL intensity of untreated control PSII (curve e) was almost the same as that after excitation at 0 °C (curve a), whereas the intensity of the upshifted TL band in low-pH-treated PSII (curve f) was markedly lower than that after excitation at 0 °C (curve b). This is due to the low efficiency of the S_1 to S_2 transition at low temperatures as reported in our previous paper (Ono & Inoue, 1990a; Ono et al., 1991). Interestingly, this low efficiency was relieved over 80% when low-pH treatment was followed by NaCl/dark washing (curve g). Note that the trough around 0 °C on glow curve g is due to the change in the heating rate due to melting of ice. As reported previously (Ono & Inoue, 1990a; Ono et al., 1991), exogenous Ca^{2+} reverses the abnormally upshifted TL peak temperature and the upshift of threshold temperature for the S_1 to S_2 transition in low-pH-treated PSII. It thus appears at a glance that removal of the two extrinsic proteins gave rise to an effect similar to exogenous Ca^{2+}. Surprisingly, however, when the low-pH NaCl/dark-treated PSII was reconstituted with the 16- and 24-kDa extrinsic proteins, the TL yield after –40 °C excitation was again suppressed (curve h). These results indicate that such abnormal properties of the S_2 state in low-pH-treated PSII as expressed by the upshifts in TL peak temperature and in threshold temperature for the S_1 to S_2 transition are not simply due to Ca^{2+} depletion or putative ligation of citrate to the Mn cluster but are contributed by the association of the two extrinsic proteins.

Figure 2 shows the effects on TL glow curves of the same three treatments as in Figure 1, but given in a reversed order: low-pH treatment was preceded by NaCl/dark washing, a reversal of the protocol in Figure 1 experiments. NaCl/dark-washed PSII showed the TL band arising from $S_2Q_A^-$ charge recombination peaking at around 15 °C after flash excitation either at 0 °C (curve b) or at –40 °C (curve g). The peak temperature did not show any upshift even though the NaCl/dark washing was followed by low-pH treatment after excitation either at 0 °C or at –40 °C (curves c and h). No significant changes in peak temperature were observed either after reconstitution with the two extrinsic proteins (curves d and i). However, when NaCl/dark–low-pH-treated PSII was further reconstituted with the two extrinsic proteins, the TL

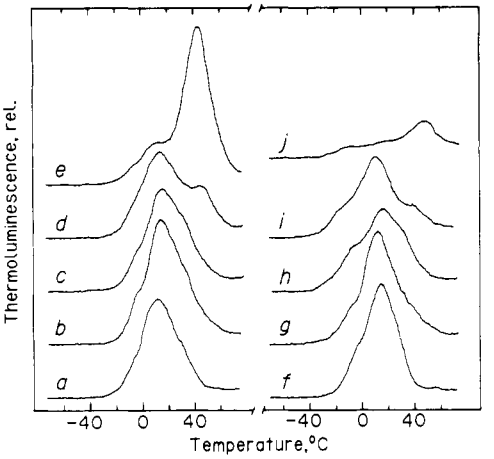

FIGURE 2: TL glow curves induced by single-flash excitation at 0 °C (left side) and at –40 °C (right side) of PSII membranes in the presence of 10 μM DCMU. Untreated control PSII (a, f); NaCl/dark-washed PSII (b, g); NaCl/dark-washed PSII followed by low-pH treatment (c, h); NaCl/dark-washed PSII followed by reconstitution with the 16- and 24-kDa extrinsic proteins (d, i); NaCl/dark-washed PSII followed by low-pH treatment and then reconstituted with the 16- and 24-kDa proteins (e, j). Saturating amounts of the two extrinsic proteins were added for protein reconstitution.

peak temperature was markedly upshifted (curves e and j), and its emission intensity was high after excitation at 0 °C (curve e) but much lower after excitation at –40 °C (curve j). From its upshifted peak temperature and its low efficiency of formation at –40 °C, this TL component found in NaCl/dark–low-pH-treated PSII after reconstitution with the two extrinsic proteins will correspond to the one that was found in low-pH-treated PSII (Figure 1b). These results again indicate that Ca^{2+} depletion in the presence, but not in the absence, of the two extrinsic proteins is the prerequisite for the abnormal S_2 state that exhibits the upshifts in TL peak temperature and threshold temperature for the S_1 to S_2 transition.

Ca^{2+} depletion by NaCl wash is known to require light (Dekker et al., 1984; Ono & Inoue, 1986; Miyao & Murata, 1986; Boussac & Rutherford, 1988a), so that the NaCl/dark-washed PSII used in this experiment would probably retain Ca^{2+}, although some O_2 centers might have lost Ca^{2+} during the treatment. We note that the small but distinct shoulder at around 40 °C in the glow curve of NaCl/dark-washed PSII after the reconstitution (curve d) would probably be contributed by these centers. It is thus very likely that the abnormal properties of the S_2 state manifest only when the 16- and/or 24-kDa proteins are retained in Ca^{2+}-depleted O_2 centers.

Table I shows the effects on O_2 evolution of various combinations of treatments involving Ca^{2+} depletion and protein reconstitution. Experiment 1 shows that NaCl/dark washing reduces the activity by half and the remaining activity is further reduced to less than 10% by the following low-pH treatment. Reconstitution with the two extrinsic proteins restores the activity in NaCl/dark-washed PSII but not in NaCl/dark–low-pH-treated PSII, whereas exogenous Ca^{2+} restores the activity in both of the two PSIIs. The fact that the reconstitution could restore nearly 80% O_2 evolution in NaCl/dark-washed PSII with no addition of exogenous Ca^{2+} implies that about 80% of the O_2 centers in this preparation retained functional Ca^{2+}. As reported previously, depletion of the 16- and 24-kDa proteins by NaCl/dark washing of normal PSII induces a heterogeneity among the Ca^{2+} pool with respect to its light susceptibility (Ono & Inoue, 1986; Cammarata & Cheniae, 1987): a part of the Ca^{2+} is tightly

Table I: Effects of NaCl/Dark Wash, Low-pH Treatment, and Extrinsic Protein Reconstitution on O_2 Evolution Activity

treatment	O_2 evolution act. no addition	O_2 evolution act. +30 mM $CaCl_2$
experiment 1		
none	100 (740)[a]	106 (790)
NaCl/dark	44 (328)	85 (655)
NaCl/dark → low pH	7 (53)	81 (596)
NaCl/dark → 16,24-kDa reconstitution	77 (572)	83 (612)
NaCl/dark → low pH → 16,24-kDa reconstitution	10 (80)	71 (524)
experiment 2		
none	100 (715)	105 (757)
low pH	11 (83)	78 (561)
low pH → NaCl/dark	8 (59)	72 (512)
low pH → NaCl/dark → 16,24-kDa reconstitution	9 (65)	73 (520)

[a] Numbers in parentheses represent the O_2 evolution activity in micromoles of O_2 per milligram of Chl per hour.

bound while the other is easily released during O_2 assay by the action of actinic light. This has been interpreted to mean that the 24-kDa protein functions as a barrier against light-triggered release of Ca^{2+}. Apparently, the activity restoration observed in experiment 1 by reconstitution with the two extrinsic proteins may be contributed by this protective function, rather than the putative conformational regulation of the Mn cluster by binding of the two extrinsic proteins. This view is compatible with the observation that no activity stimulation could be found by protein reconstitution to NaCl/dark–low-pH-treated PSII which is completely depleted of Ca^{2+}. This indicates in turn that the inhibition by low-pH treatment is directly due to depletion of Ca^{2+}.

As shown by experiment 2, the inhibition of O_2 evolution induced by low-pH treatment could not be restored either by NaCl/dark washing or by the following reconstitution with the two extrinsic proteins, whereas largely restored by exogenous Ca^{2+}. It is thus inferred that O_2 evolution is inhibited regardless of the presence or absence of the two extrinsic proteins when Ca^{2+} is depleted, in agreement with the results of experiment 1. The absolute requirement of Ca^{2+} for O_2 evolution again confirms that reversion of the abnormally upshifted TL band in low-pH-treated PSII to a nearly normal TL band upon the following NaCl/dark washing cannot be due to a fortuitous reconstitution of functional Ca^{2+}.

Figure 3 shows the dependence on excitation temperature of the S_1 to S_2 transition in variously treated PSII preparations. Samples were illuminated with a single saturating flash at varying temperatures, and the heights of normal or abnormally upshifted TL glow peaks were plotted against excitation temperature. Consistent with our previous report (Ono & Inoue, 1990a; Ono et al., 1991), the threshold temperature for the S_1 to S_2 transition in low-pH-treated PSII was markedly upshifted (open squares) as compared with that in untreated control PSII (open circles), and the upshift was largely reversed when low-pH treatment was followed by NaCl/dark washing (closed triangles), although a slight irreversible upshift remained. Similar irreversibility was also observed for NaCl/dark-washed PSII (open triangles) that retained almost all the functional Ca^{2+} (see Table I). Presumably, these would be due to unidentified irreversible damage brought about by exposure to high salt. Notably, however, when the two extrinsic proteins were rebound to low-pH NaCl/dark-treated PSII, the threshold temperature for the S_1 to S_2 transitions was again markedly upshifted to exhibit the same temperature dependency as found for low-pH-treated PSII (closed squares).

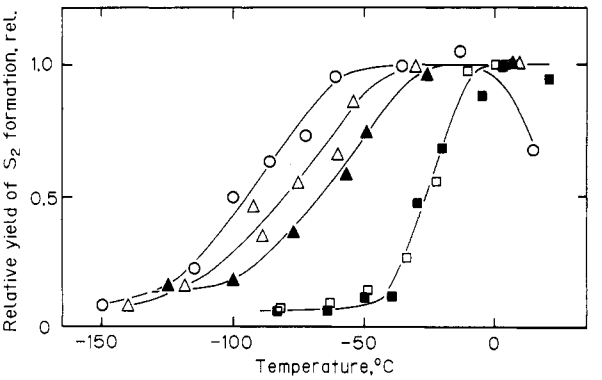

FIGURE 3: Temperature dependence of the S_1 to S_2 transition monitored by the intensity of the TL band. Sample PSII membranes were excited by a single flash in the presence of 10 μM DCMU at varying temperatures, and the intensities of the TL glow peak were plotted against excitation temperature. Untreated control PSII (○); low-pH-treated PSII (□); low-pH-treated PSII followed by NaCl/dark washing (▲); NaCl/dark-washed PSII (△); low-pH-treated PSII followed by NaCl/dark washing and then reconstituted with the 16- and 24-kDa extrinsic protins (■). Saturating amounts of the two extrinsic proteins were added for protein reconstitution. The TL intensities were expressed in percent relative to the maximum heights of glow peaks of respective PSII samples. Half-inhibition temperatures for the S_1 to S_2 transition are listed in Table II.

Table II: Effects of NaCl/Dark Wash, Low-pH Treatment, and Extrinsic Protein Reconstitution on Half-Inhibition Temperatures for S_2 Formation, the Presence of Functional Ca, the Presence of 16- and 24-kDa Proteins, and O_2 Evolution Activity

treatment	half-inhibn temp (°C)	Ca[a]	16,24-kDa proteins	O_2 evolution
none	−94	+	+	+
NaCl/dark	−76	+	−	+
low pH	−25	−	+	−
low pH → NaCl/dark	−65	−	−	−
low pH → NaCl/dark → 16,24-kDa reconstitution	−25	−	+	−
low pH → Ca^{2+} addition[b]	−90	+	+	+

[a] The presence of functional Ca was estimated from the O_2 evolution activity in the absence of externally added Ca^{2+}. [b] Data taken from Ono and Inoue (1988, 1990a).

Table II summarizes the threshold temperatures for the S_1 to S_2 transition in variously treated PSII preparations. The table also lists whether the preparations were sufficient or deficient with respect to the extrinsic proteins and functional Ca^{2+}. The data clearly show that the upshift of threshold temperature manifests only when the PSII samples are depleted of functional Ca^{2+} but retain the two extrinsic proteins. Notably, the absence of functional Ca^{2+} does not much affect the threshold temperature when the two extrinsic proteins are absent.

On the basis of these data, we may consider that association of two extrinsic proteins with the O_2 centers is indispensable for the expression of the abnormal properties of the Ca^{2+}-depleted S_2 state. We attempted to identify which of the two proteins was responsible for this phenomenon. PSII membranes were first subjected to low-pH treatment for complete depletion of Ca^{2+} and then washed with various concentrations of NaCl in the dark to partially remove the extrinsic proteins. The Ca^{2+}-depleted PSII retaining varied amounts of two extrinsic proteins was then subjected to examinations of TL (Figure 4) and protein compositions (Figure 5). As shown in Figure 4, the TL component exhibiting the upshifted peak temperature characteristic of the low-pH-treated PSII grad-

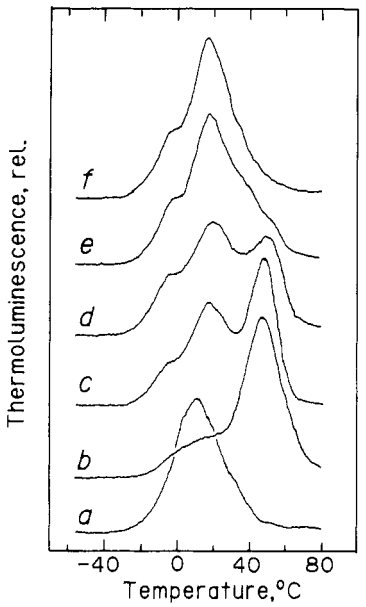

FIGURE 4: Effects of removal of the 16- and 24-kDa extrinsic proteins on the peak temperature of TL after single-flash excitation at 0 °C in the presence of 10 μM DCMU. Untreated PSII (a); low-pH-treated PSII followed by NaCl/dark washing at 0.02 (b), 0.2 (c), 0.4 (d), 1 (e), and 1.5 M (f).

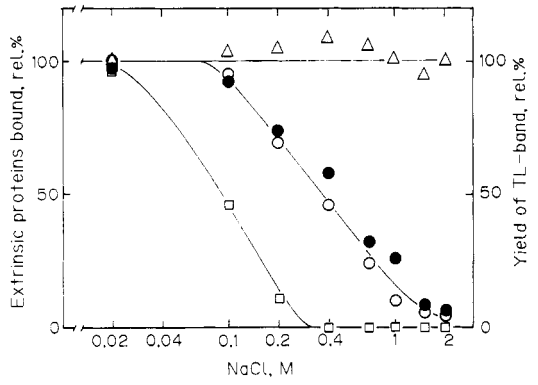

FIGURE 5: Dependence on the salt concentration during NaCl/dark washing of the amplitude of the high peak temperature TL component and the amount of three (16, 24, and 33 kDa) extrinsic proteins retained in PSII membranes. Low-pH-treated PSII membranes were further subjected to NaCl/dark washing at varying concentrations of NaCl. The amount of 16- (□), 24- (○), and 33-kDa (△) proteins retained in PSII and the amplitude of the high peak temperature TL component (●) estimated from the glow curves in Figure 4 were plotted against the NaCl concentration. Both the protein amounts and the TL amplitude were expressed in percent relative to those in the original low-pH-treated PSII. The samples were excited by a single flash at 0 °C in the presence of 10 μM DCMU.

ually decreased with increasing salt concentration during the NaCl/dark wash, being accompanied by a gradual increase in the amplitude of the normal peak temperature TL component characteristic of the extrinsic protein-depleted PSII. The heights of the two glow peaks were approximately the same in PSII washed with 0.4 M NaCl, while after being washed with 1.5 M NaCl, only one component was observed at around 16 °C with no 43 °C component remaining.

Figure 5 shows the effect of NaCl concentration on removal of the three extrinsic proteins. The 16-kDa (open squares) and 24-kDa (open circles) proteins began to dissociate upon washing with 20 and 100 mM NaCl and were almost lost at 0.4 and 1.5 M NaCl, respectively, but the 33-kDa proteins (open triangles) were not removed at any NaCl concentrations tested. When the relative intensity of the upshifted TL component (see Figure 4) was plotted in the same figure (solid

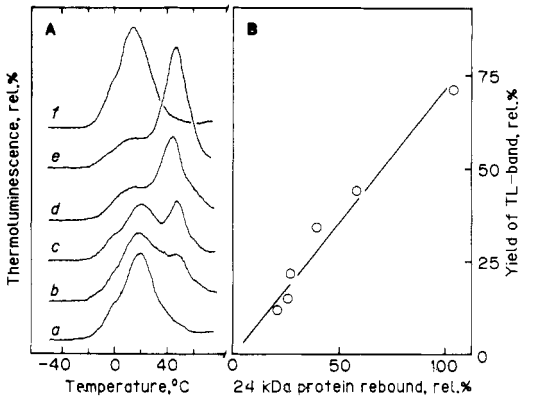

FIGURE 6: Quantitative relationship between the amount of PSII-bound 24-kDa extrinsic protein and the ampitude of the abnormally upshifted TL glow peak. Low-pH-treated PSII was further washed by 2 M NaCl in the dark and then reconstituted with various amounts of 24-kDa protein. (A) TL glow curves of various PSII membranes: untreated control PSII (f); low-pH-treated PSII (e); low-pH-treated PSII followed by NaCl/dark washing (a); low-pH-treated PSII followed by NaCl/dark washing and then reconstituted with limited amounts of 24-kDa protein (b, c) or with an excess amount of 24-kDa protein (d). (B) Relationship between the relative yield of the high peak temperature TL component and the relative amount of 24-kDa protein associated with PSII. The relative yield of the high peak temperature TL component was estimated from the peak height at 43 °C of TL glow curves in (A). The protein amount and the TL amplitude were expressed in percent relative to those obtained for the original low-pH-treated PSII. The samples were excited by a single flash at 0 °C in the presence of 10 μM DCMU.

circles), it closely coincided with the abundance of the 24-kDa protein remaining associated with PSII, but not with the abundance of the 16-kDa protein. We note that the NaCl concentrations required for liberation of the two extrinsic proteins are almost the same between low-pH-treated PSII and untreated control PSII (Ono and Inoue, unpublished data), suggesting that the binding affinities of these two proteins do not much depend on the presence or absence of functional Ca^{2+}. This indicates that the extrinsic proteins in low-pH-treated samples are associated with PSII at their proper binding sites, although they are known to be once dissociated from PSII during the treatment but rebind again on adjustment of pH (Shen & Katoh, 1991).

In Figure 6, the effect of protein association on the TL glow curve was more precisely examined by reconstitution with a purified extrinsic protein. Chromatographically purified 24-kDa protein was added to low-pH NaCl/dark-treated PSII in which both the functional Ca^{2+} and the two extrinsic proteins were depleted. As shown in the left panel, low-pH NaCl/dark-treated PSII showed a TL band at around 18 °C. Upon reconstitution with the 24-kDa protein, the intensity of the TL band peaking at around 18 °C decreased while that of the band peaking at 43 °C increased, showing a linear relation between the relative amount of the 24-kDa protein rebound and the relative amplitude of the 43 °C TL component (Figure 6, right panel): the 43 °C TL component was originally found in low-pH-treated PSII, but was lost after NaCl/dark washing, and was again regenerated by 70–75% when the 24-kDa protein was fully reconstituted. The incomplete regeneration may be attributable to a small fraction of O_2 centers that were irreversibly damaged to lose the capability of TL emission during the depletion and reconstitution procedures. On the basis of the results in Figures 5 and 6, we may conclude that association of the 24-kDa protein to Ca^{2+}-depleted O_2 centers is the exclusive cause for the abnormal properties of the modified S_2 state.

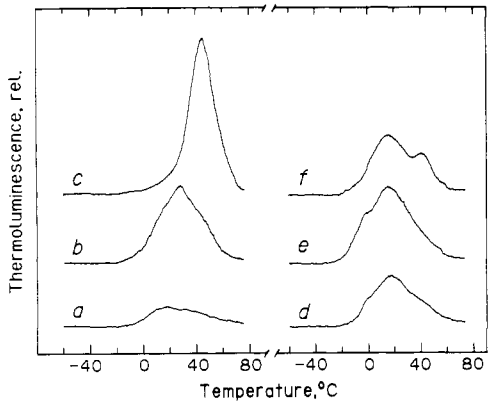

FIGURE 7: TL glow curves of NaCl/light/EDTA-washed PSII membranes induced by continuous illumination at 77 K for 30 s (left side) or by single-flash excitation at 0 °C (right side) is the presence of 10 μM DCMU. Untreated control PSII (a, d); NaCl/light/EDTA-washed PSII (b, e); NaCl/light/EDTA-washed PSII followed by reconstitution with 16- and 24-kDa extrinsic proteins (c, f).

As shown in Table I, NaCl/dark-washed PSII retains functional Ca^{2+}. When the PSII membranes were washed with 2 M NaCl under illumination followed by EDTA addition, however, most of the functional Ca^{2+} is effectively removed. In this PSII (denoted as NaCl/light/EDTA-washed PSII), an abnormally dark-stable S_2 state with an extremely long life is detected (Ono & Inoue, 1990b). We examined the effect of reconstitution with the 24-kDa protein on this abnormal S_2 state. The results are shown in Figure 7. As reported previously (Ono & Inoue, 1990b), the dark-stable S_2 state shows a TL band when given an electron on Q_A by illumination at 77 K that delivers one electron from the PSII donor side to Q_A without affecting the S state (Rutherford et al., 1982; Ono & Inoue, 1990b). Note that Q_A in the treated PSII keeps the oxidized form after dark relaxation. Given on Q_A a negative counterpart, the positive charge of the dark-stable S_2 state undergoes charge recombination to emit a TL band at 25 °C (curve b). Interestingly, this TL band resembles those found in low-pH NaCl/dark-treated PSII (see Figure 1) or those in NaCl/dark–low-pH-treated PSII (see Figure 2), although the peak temperature was slightly elevated. This slight upshift is presumably due to preferential loss of the charge pair emitting TL at lower temperatures during dark incubation at 0 °C. If we assume that the abnormal properties of the Ca^{2+}-depleted S_2 state are commonly regulated by the association of the 24-kDa extrinsic protein, we may expect that this TL band will be converted to show an upshifted peak temperature upon reconstitution with the 24-kDa protein. As shown by glow curve c, when the NaCl/light/EDTA-washed PSII was subjected to TL measurement after reconstitution with the 24-kDa extrinsic protein, a TL band having an elevated peak temperature was generated. This TL band is quite similar to the one that is induced in low-pH-treated PSII, except for the differences in the illumination condition: the former required 77 K continuous light illumination to provide only the negative charge, indicating the retention of the dark-stable S_2 state during and after the reconstitution procedures, while the latter required one flash to generate a charge pair at 0 °C, the excitation temperature above the upshifted threshold temperature (see Figure 1).

The right panel of Figure 7 shows the TL glow curves induced by a single-flash excitation at 0 °C of NaCl/light/EDTA-treated PSII that retained the dark-stable S_2 state. Interestingly, a TL band peaking at around 10 °C was generated either before or after reconstitution with the 24-kDa protein. As reported previously, this TL component is attributed to a charge recombination involving a redox component detected as a $g = 2.0$ EPR signal that is presumed to arise from an organic radical stabilized on the donor side of PSII (Boussac et al., 1989; Sivaraja et al., 1989; Ono & Inoue, 1990b). Our present result, therefore, suggests that the TL properties of this radical component are not affected by binding of the 24-kDa protein, although this TL band is specific to Ca^{2+}-depleted PSII. Presumably, binding of the 24-kDa protein does not alter the redox properties of either the positive charge on the putative organic radical or the negative charge on Q_A^-.

Table III: Effect of NaCl/EDTA/Light Treatment and Extrinsic Protein Reconstitution on O_2 Evolution Activity

treatment	O_2 evolution act.	
	no addition	+30 mM $CaCl_2$
none	100 (682)[a]	113 (772)
NaCl/light/EDTA	9 (63)	93 (632)
NaCl/light/EDTA → 16,24-kDa reconstitution	14 (100)	71 (482)

[a] Numbers in parentheses represent the O_2 evolution activity in micromoles of O_2 per milligram of Chl per hour.

Table III shows the effects of reconstitution with the 24-kDa protein on O_2 evolution by NaCl/light/EDTA-washed PSII. The activity was markedly inhibited after NaCl/light/EDTA washing, and reconstitution with the two extrinsic proteins did not restore the activity either. However, the activity was largely restored by the addition of exogenous Ca^{2+} in both PSII preparations regardless of the presence or absence of the two extrinsic proteins. These indicate that functional Ca^{2+} was equally depleted in both of these preparations.

DISCUSSION

The present results showed that the Ca^{2+}-depleted water oxidation system exhibits marked modifications in its S_2-state properties only when the 24-kDa extrinsic protein binds to PSII. The modifications are characterized by an abnormal TL band with an elevated peak temperature and an abnormal temperature dependence for the S_1 to S_2 transition with an extremely upshifted threshold temperature. Notably, these abnormalities do not manifest themselves if the Ca^{2+}-depleted PSII is devoid of the 24-kDa protein, although Ca^{2+} depletion inhibits O_2 evolution regardless of the presence or absence of this extrinsic protein. In turn, these abnormalities once manifested are reversed to normal without restoration of O_2 evolution when this extrinsic protein is dissociated from PSII, and are again induced when reconstituted with the extrinsic protein. During these reversible interchanges, the relative extents of the abnormalities show a linear relationship with the amount of the extrinsic protein associated with PSII (Figure 6). The result suggests that an all-or-none-type modification occurs in individual Ca^{2+}-depleted O_2 centers by binding a single molecule of the 24-kDa protein at its proper binding site in PSII. This idea is consistent with the stoichiometric rebinding of the 24-kDa protein to the PSII complex (Miyao & Murata, 1989) with the same affinity regardless of the presence or absence of bound Ca^{2+} (this study). The fact that the redox properties of the Mn cluster in Ca^{2+}-depleted PSII depend upon the 24-kDa protein suggests that this protein closely interacts not only with Ca^{2+} but also with the Mn cluster. This view is compatible with the reports that stable binding of the 24-kDa protein requires the Mn cluster (Becker et al., 1985; Ono et al., 1986; Kavelaki & Ghanotakis, 1991) and that removal of the two extrinsic proteins makes the Mn

cluster accessible to bulky exogenous reductants which destroy the Mn cluster (Ghanotakis et al., 1984b). The presence of a Ca atom in close proximity of the Mn cluster has also been proposed on the basis of EXAFS (extended X-ray absorption fine structure) data (Penner-Hahn et al., 1990).

In previous papers (Ono & Inoue, 1990a; Ono et al., 1991), we reported that Ca^{2+} depletion by low-pH treatment of PSII induces a modified S_2 state which exhibits an elevated TL peak temperature and a narrowed EPR multiline signal accompanied by an extremely upshifted threshold temperature for their induction. Structural modulation of the Mn cluster in this S_2 state could be evidenced more directly by an XANES (X-ray absorption near-edge structure) spectrum: the K-edge energy is downshifted in the absence of Ca^{2+}, but is reversed to normal by addition of Ca^{2+} accompanied by restoration of O_2 evolution (Ono et al., 1991). We interpreted these results to mean that the structural modulations of the Mn cluster as evidenced by the K-edge energy downshift are due simply to the absence of Ca^{2+}: no effect of the 24-kDa protein was taken into account. In the present study, however, it was clearly shown that a similarly abnormal S_2 state could be found in NaCl/light/EDTA-washed PSII, if only the Ca^{2+}-depleted PSII was reconstituted with the 24-kDa protein. This indicates that not only Ca^{2+} depletion but also the presence of the 24-kDa protein are the important requirements for the manifestation of these abnormalities. In view of this fact, we may possibly assume that the above mentioned K-edge downshift is contributed by the retention of the extrinsic protein in low-pH-treated PSII, although we have at present no experimental evidence for this particular point.

A speculative explanation for these phenomena may be given as follows: the 24-kDa extrinsic protein interacts with an intrinsic protein(s) of PSII that ligate(s) both Ca and Mn. This intrinsic protein takes two different conformations depending on the presence or absence of bound Ca^{2+}, and the 24-kDa extrinsic protein reinforces both of the two conformations: the reinforcement in the presence of Ca^{2+} stabilizes the bound Ca^{2+} to be resistant to light-induced release, and the reinforcement in the absence of Ca^{2+} constitutes a higher barrier for exogenous Ca^{2+} in getting access to its binding site. Upon depletion of both Ca^{2+} and the 24-kDa extrinsic protein, this reinforcement disappears, so that the Mn-binding intrinsic protein will take a relaxed form with a rather normal structure of the Mn cluster that resembles the normal, active form. This corresponds to NaCl/light/EDTA washing. Upon depletion of Ca^{2+} while leaving the 24-kDa extrinsic protein remaining associated, the structural reinforcement for the Mn-binding intrinsic protein persists, resulting in so strongly a constrained structure of the Mn-binding intrinsic protein that the Mn cluster ligating on this protein may exhibit such abnormal properties as observed in this and previous studies. This corresponds to low-pH treatment or NaCl/light/EDTA washing followed by reconstitution with the 24-kDa extrinsic protein. Presumably, the structural reinforcement by the 24-kDa protein will facilitate the Mn-cluster-ligating protein(s) to take a conformation suitable for catalyzing O_2 evolution in concert with Ca^{2+}, but only when the functional Ca^{2+} is depleted, the reinforcement functions to exaggerate the slight conformation changes in the vicinity of the Mn cluster induced by the absence of Ca^{2+}, thereby resulting in such a structurally constrained conformation of the Mn cluster. In this model, we assumed that the 24-kDa protein binds directly to a Mn-cluster-ligating intrinsic protein. The same, but an indirect, modulation of the Mn cluster will also be possible if we assume that the 24-kDa protein binds to the 33-kDa extrinsic protein as previously suggested (Miyao & Murata, 1989; Kavelaki & Ghanotakis, 1991).

The extreme upshift of the threshold temperature for the S_1 to S_2 transition is one of the striking features of the phenomena. Generally, the temperature dependence of the S_1 to S_2 transition reflects the rate of electron transfer from the Mn cluster in the S_1 state to Z^+ (oxidized form of the secondary electron donor to P_{680}). Thus, the upshift in threshold temperature will imply an increase in the activation energy of the S_1 to S_2 transition. The upshift of the TL peak temperature will in turn imply an increase in the activation energy of the back-reaction from S_2 to S_1, if we assume that the electron transfer on the donor side of PSII is the major limiting factor during the recombination for TL emission. Presumably, binding of the 24-kDa protein to the Ca^{2+}-depleted O_2 center increases the energetic barrier between S_1 and S_2. It is noted, however, that the 24-kDa protein-dependent abnormal properties of the Mn cluster are not always directly responsible for the incapability of O_2 evolution of Ca^{2+}-depleted O_2 centers: the abnormal properties are largely reversed by removal of the 24-kDa protein without restoring O_2 evolution, and restoration of O_2 evolution definitely requires reconstitution with Ca^{2+}. Regarding this point, a slight difference in TL properties can be seen between the PSII depleted of both Ca^{2+} and 24-kDa protein and the normal O_2-evolving PSII: the TL peak temperature of the former PSII is slightly but reproducibly higher by 5 °C than that of the latter PSII.

There are many papers discussing the role of Ca^{2+} in O_2 evolution, in which variously divergent procedures for Ca^{2+} extraction have been used [for a review, see Yocum (1991)]. In most of these studies, however, little attention has been paid to this point, whether or not the 24-kDa protein is preserved after depletion of Ca^{2+}. Seemingly, many of the reported results need to be reconsidered by taking into account the significant influence of the 24-kDa-protein. Three procedures have been routinely applied for preparing Ca^{2+}-depleted PSII: (i) 1–2 M NaCl washing in the dark (Ghanotakis et al., 1984a,b; Miyao & Murata, 1984; Franzen et al., 1985; Ono & Inoue, 1986; de Paula et al., 1986; Cammarata & Cheniae, 1987); (ii) 1–2 M NaCl washing in the light plus chelator (EDTA or EGTA) followed by reconstitution with the 16- and 24-kDa proteins (Boussac et al., 1985, 1989, 1990a,b; Miyao & Murata, 1986) or no reconstitution (Boussac et al., 1985; Boussac & Rutherford, 1988b; Ono & Inoue, 1990b), and (iii) low-pH treatment in the dark (Ono & Inoue, 1988, 1989a; Ono et al., 1991; Sivaraja et al., 1989). Among these three, procedure i, mostly used in earlier works, is the least stringent treatment, since light and/or chelator is additionally required for complete suppression of O_2 evolution. The latter two procedures are surer with respect to the completeness of Ca^{2+} depletion, provided the concentration of the chelator is high enough to eliminate contaminating free Ca^{2+} (Ono & Inoue, 1990c). As evidenced by the present study, however, the structural and functional properties of the Mn cluster differ markedly depending on the presence and absence of the 24-kDa protein, and this may explain some of the inconsistent observations in literature: e.g., the $g = 2$ EPR signal was induced at the expense of the dark-stable modified S_2 EPR-multiline signal upon illumination of the Ca^{2+}-depleted PSII prepared by NaCl/EGTA/light washing followed by reconstitution with the 16- and 24-kDa proteins (Boussac et al., 1989, 1990a,b) and by low-pH treatment (Sivaraja et al., 1989), whereas these two EPR signals coexisted

in the Ca^{2+}-depleted PSII prepared by NaCl/light/EDTA washing with no reconstitution with the two extrinsic proteins (Ono & Inoue, 1990b). Presumably, these differences are due to the differences in magnetic properties of the Mn cluster in the presence (former PSII) or absence (latter PSII) of the 24-kDa protein, since the EPR signal is proposed to arise from an organic radical, presumed to be histidine, under the influence of magnetic interaction with the Mn cluster (Boussac et al., 1990a).

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