Structural and Functional Modifications of the Manganese Cluster in Ca^{2+}-Depleted S_1 and S_2 States: Electron Paramagnetic Resonance and X-ray Absorption Spectroscopy Studies†

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ABSTRACT: The effect of extraction of weakly bound Ca^{2+} by low-pH treatment on the O_2-evolving apparatus was studied by use of low-temperature electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy. In low-pH-treated PSII membranes, an S_2 EPR multiline signal with modified line shape was induced by illumination at 0 °C, but its signal amplitude decreased upon lowering the excitation temperature with concomitant oxidation of cytochrome (cyt) *b*-559 in place of Mn. The half-inhibition temperature for formation of the modified multiline signal was found at −33 °C, which was much higher than that for formation of the normal S_2 state in untreated control membranes. Signal II_f was normally induced down to −30 °C, but its dependence on excitation temperature was different from that for modified S_2. This was interpreted as indicating that the low-temperature blockage of modified S_2 formation is due to the incapability of electron abstraction from the Mn cluster. The Mn K-edge of X-ray absorption near-edge structure (XANES) spectrum shifted to lower energy by 0.8 eV after low-pH treatment, but the shift was reversed by addition of Ca^{2+}. Upon illumination at 0 °C of treated membranes, the K-edge energy was up-shifted by 0.8 eV, but was not upon illumination at 210 K. These results were interpreted as indicating that extraction of weakly bound Ca^{2+} by low-pH treatment gives rise to structural and functional modulations of the Mn cluster.

Evidence recently accumulated strongly indicates that water is photosynthetically oxidized in a catalytic center consisting of four Mn atoms which probably assemble to form a tetranuclear cluster possibly ligating to an apoprotein(s) through carboxyl and/or imidazole moieties of amino acid residues. This Mn center is capable of cycling through five distinct oxidation states labeled S_0–S_4 (Kok et al., 1970). Among the four light-driven transition steps, the event of the S_1 to S_2 transition has been studied most intensively. Illumination at low temperature accumulates the S_2 state which reveals electron paramagnetic resonance (EPR)[1] signals arising from magnetic interactions between the Mn atoms. Results obtained from X-ray absorption near-edge structure (XANES)[1] and EPR studies indicated that the Mn atom is actually oxidized upon the S_1 to S_2 transition. This step has been known to be unique among the four transitions in that it does not involve proton release, and consequently the formal charge of the Mn cluster increases from 0 to 1 [for reviews, see Dismukes (1986), Brudvig et al. (1989), and Babcock et al. (1989)]. Interestingly, the S_1 to S_2 transition persists to much lower temperature than the other transition steps.

Recent observations indicate that this transition process is largely affected by such treatments as NaCl/chelator wash (Boussac et al., 1989; Ono & Inoue, 1990b) or low-pH treatment (Ono & Inoue, 1989a,b, 1990a; Sivaraja et al., 1989), both of which are claimed to release weakly bound Ca^{2+} from the O_2-evolving apparatus. On illumination of these treated membranes, an altered S_2 as depicted by a modified multiline signal and an up-shifted thermoluminescence band is generated. When the PSII membranes bearing such altered S_2 are further illuminated, an extra positive charge accumlates in Ca^{2+}-depleted O_2 centers, exhibiting a 163-G-wide EPR signal at $g = 2.004$ (Boussac et al., 1989; Sivaragja et al., 1989; Ono & Inoue, 1990b) or a new thermoluminescence band peaking around 10 °C (Ono & Inoue, 1990b), which are claimed to be an oxidized histidine (Boussac et al., 1990; Ono & Inoue, 1990b). Recently, we have reported that the modified S_2 multiline signal is not generated by illumination at −60 °C but is efficiently generated by illumination at −5 °C (Ono & Inoue, 1990a). Temperature dependence studies by use of thermoluminescence suggested that the lower limiting temperature for S_2 formation is markedly elevated in treated membranes.

In the present study, we tried to characterize the putative modifications of the Mn cluster in the S_1 and S_2 states caused by low-pH treatment by means of EPR and XANES spectroscopy. The results indicate a structural modulation of the Ca^{2+}-depleted Mn cluster and confirm that electron abstraction

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[1] Abbreviations: Chl, chlorophyll; cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHCP, light-harvesting chlorophyll protein; EPR, electron paramagnetic resonance; MES, 4-morpholineethanesulfonic acid; PS, photosystem; Q_A, primary quinone acceptor of photosystem II; Y_D, auxiliary electron donor of photosystem II; Y_Z, secondary electron donor of photosystem II; XANES, X-ray absorption near-edge structure.

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from the Mn cluster in the modified S_1 state requires a higher temperature than that in normal S_1.

Materials and Methods

PSII membranes capable of O_2 evolution were prepared from spinach by solubilization with Triton X-100 (Berthold et al., 1981) and stored in liquid N_2. After being thawed, the membranes were incubated in darkness for 6 h to afford complete relaxation of the acceptor and donor sides of PSII. The following treatments were performed under a dim green safe light unless otherwise noted. The dark-adapted membranes were washed once with 400 mM sucrose, 20 mM NaCl, 1 mM EDTA, and 40 mM MES/NaOH (pH 6.5) followed by two washes with the same medium lacking EDTA. Then the membranes were subjected to low-pH treatment by incubation with 400 mM sucrose, 20 mM NaCl, and 20 mM citrate/NaOH (pH 3.0) in complete darkness at 0 °C for 5 min as described previously (Ono & Inoue, 1988), or to NH_2OH treatment by incubation with 400 mM sucrose, 20 mM NaCl, 1 mM NH_2OH, and 40 mM MES/NaOH (pH 6.5) at 0.5 mg of Chl/mL for 1 min at 0 °C in darkness after one wash with 2 M NaCl. The treated membranes were centrifuged and resuspended in 400 mM sucrose, 20 mM NaCl, and 40 mM MES/NaOH (pH 6.5) supplemented with 0.05 mM DCMU (10 mM dimethyl sulfoxide solution as stock) unless otherwise noted.

For measurements of the multiline EPR signal, the sample was placed in a calibrated quartz EPR tube, frozen in an ethanol/dry ice bath, and stored in liquid N_2. All procedures were carried out within 30 min after low-pH treatment. For Ca^{2+} repletion, the low-pH-treated membranes were incubated in darkness for 30 min at 0 °C after pH adjustment, supplemented with 50 mM $CaCl_2$, followed by 30-min dark incubation at 0 °C. EPR samples were preincubated at the designated temperature for 4 min, then illuminated with a pair of 600-W tungsten lamps from both sides (30 s, −5 °C; 2 min, between −30 and −70 °C; 4 min, between −80 and −180 °C), and then cooled to 77 K by dipping into liquid N_2 unless otherwise noted. The sample temperature was controlled within ±0.5 °C during illumination by use of a JEOL ES DVT-1 temperature control system. EPR signals were recorded at 6 K within 1 h after illumination as described previously (Ono & Inoue, 1989b). A JEOL ES-PRIT 23 EPR data system was used for averaging and subtraction of spectra.

For measurements of signal II, low-pH-treated or NH_2OH-treated membranes were preilluminated for 1 s to oxidize Y_D, incubated in darkness at 0 °C for 1 min, and then kept in liquid N_2. The samples were warmed and incubated at the designated temperatures for 4 min in an EPR cavity equipped with a continuous-flow cryostat (JEOL ES DVT-1), and an EPR signal was recorded before (signal II_S) and during (signal II_{S+f}) illumination. DCMU was not included in the sample to allow multiple turnovers of the PSII reaction center.

For XANES measurements, low-pH-treated membranes or Ca^{2+}-repleted membranes were supplemented with 0.05 mM DCMU and centrifuged at 35000g for 35 min after pH adjustment, and the resulting pellet was directly applied onto a sample holder, a Cu plate 0.2 mm in thickness with a square hole in the center designed for X-ray spectroscopy by the fluorescence detection mode. The sample membranes in the holder were stored in liquid N_2 without illumination, or alternatively after illumination at 0 °C in an ice/water bath for 2.5 min to effect the S_1 to S_2 transition. All these procedures were carried out within 1 h after low-pH treatment. The sample membranes were also illuminated in a dry ice/ethanol bath at 210 K for 15 min and then stored in liquid N_2 as described (Kusunoki et al., 1990). For Ca^{2+} repletion, the treated membranes were incubated in darkness at 0 °C for 1 h after pH adjustment, supplemented with 50 mM $CaCl_2$, and then incubated in darkness at 0 °C for 30 min followed by addition of 0.05 mM DCMU.

XANES spectra were measured at the Photon Factory of National Laboratory of High Energy Physics, Tsukuba, using a sagittally shaped Si(III) crystal monochromator by the fluorescence detection mode with an EG & G modified Si(Li) solid-state detector (Oyanagi et al., 1985). Saturation of the detector by too frequent photon counts was carefully avoided. Sample temperature was controlled at 100 K during the measurement. The typical energy resolution of the monochromator at the Mn K-edge was ±0.1 eV. The obtained spectra were normalized by means of an integral-equilizing method as described (Kusunoki et al., 1990). Energy calibration was done by the sharp pre-edge peak of the $KMnO_4$ solution at 6543.3 eV. Five scans were accumulated.

Results

Figure 1 shows the low-temperature EPR spectra (light − dark) induced by continuous illumination of PSII membranes at four different temperatures. In untreated membranes (panel A), the multiline signal centering at $g = 2.0$ and a broad signal centering at $g = 4.1$, both arising from the S_2-state Mn cluster (Dismukes & Siderer, 1981; Zimmermann & Rutherford, 1986), were fully induced by illumination at −5, −40, and −70 °C, whereas at −115 °C only the $g = 4.1$ signal was generated as reported by Casey and Sauer (1984). A $g = 3.0$ signal due to oxidized cyt b-559 was appreciably photoinduced at this low temperature, indicative of partial interruption of electron transport from the Mn cluster to Y_Z.

In low-pH-treated membranes (panel B), neither the multiline nor the $g = 4.1$ signals were induced by illumination at −70 °C, in agreement with our previous results (Ono & Inoue, 1990a). In nonilluminated membranes, only signal II_S arising from Y_D^+ was detected, but no other signal around $g = 2$ (data not shown). Notably, however, the $g = 3.0$ signal was significantly generated by −70 °C illumination, indicative of appreciable oxidation of cyt b-559 in place of Mn. The intensity of the cyt b-559 signal considerably fluctuated depending on sample batches. This is probably due to differences in the amount of cyt b-559 retaining the high-potential form after low-pH treatment (data not shown). When the illumination temperature was raised above −40 °C, the multiline signal was appreciably induced, and its signal amplitude increased with temperature. However, formation of the $g = 4.1$ signal remained suppresssed at any excitation temperature. The line shape of the multiline signal induced in treated membranes was modified, showing a different hyperfine structure as depicted by a reduced line spacing in agreement with previously reported results (Sivaraja et al., 1989; Ono & Inoue, 1990a). The average line spacing on the modified spectrum was 61 and 72 G below (lower magnetic field) and above (higher magnetic field) $g = 2$, respectively. These features of modification notably resemble those induced by Sr^{2+} substitution of the Ca^{2+} binding site (Boussac & Rutherford, 1988; Ono & Inoue, 1989b) with the exception of a clear difference in threshold temperature: the present signal was not induced below −70 °C, whereas in the case of Sr^{2+} substitution, the modified signal could be induced at 200 K. In treated membranes, the cyt b-559 signal was photoinduced partially at −5 °C, suggesting that the Mn complex in some of the treated PSII centers cannot be oxidized even at this high temperature (also see Figure 2). This partial interruption of Mn oxidiation does not seem to be due to a nonspecific de-

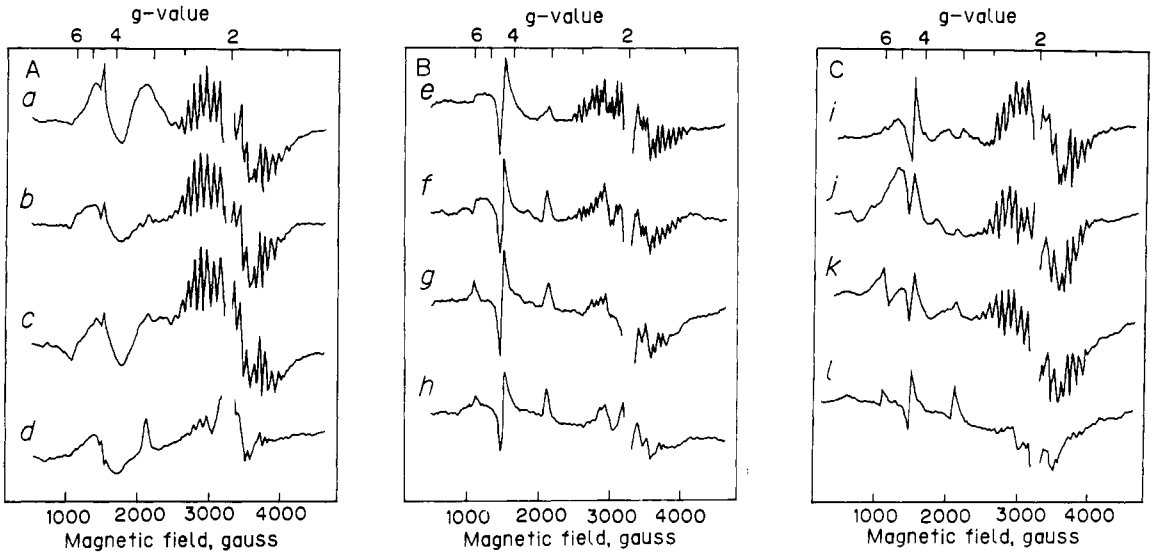

FIGURE 1: Effect of illumination temperature on low-temperature EPR spectra (light − dark) of PSII membranes. (A) Untreated control PSII membranes; (B) low-pH-treated PSII membranes; (C) low-pH-treated and then Ca^{2+}-repleted PSII membranes. Samples were illuminated with continuous light at various temperatures: −5 °C (a, e, i); −25 °C (f); −40 °C (b, g, j); −70 °C (c, h, k); and −155 °C (d, l) (30 s above −25 °C, 2 min between −30 and −70 °C, 4 min below −80 °C). Illuminated samples were rapidly cooled to 77 K. Sample concentrations were 3.8, 3.8, and 4.0 mg of Chl/mL for panels A, B, and C, respectively. Instrumental settings: temperature, 5 K; microwave power, 0.4 mW; microwave frequency, 8.95 GHz; modulation frequency and amplitude, 100 kHz and 20 G, respectively.

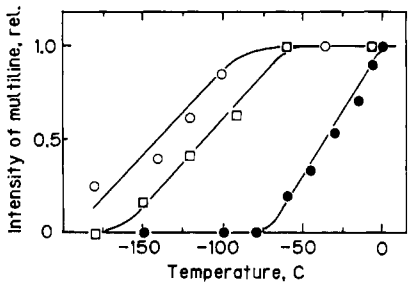

FIGURE 2: Temperature dependence of the S_1 to S_2 transition monitored by the intensity of the EPR multiline signal. Untreated control membranes (○); low-pH-treated PSII membranes (●); low-pH-treated and then Ca^{2+}-repleted PSII membranes (□). Conditions for EPR measurements are the same as in Figure 1. The signal intensities were expressed as percent relative to the maximum amplitudes of three respective samples. Half-inhibition temperatures for S_2 formation are listed in Table I.

Table I: Half-Inhibition Temperatures (°C) of the S_1 to S_2 Transition Estimated from EPR Multiline and Thermoluminescence (Ono & Inoue, 1990a)

PSII membranes	detection method: EPR multiline	detection method: thermoluminescence
untreated control	−140	−90
low-pH-treated	−33	−23
low-pH-treated and then Ca^{2+}-repleted	−110	−90

terioration of O_2 centers by the treatment, since in Ca^{2+}-repleted membranes, the cyt *b*-559 oxidation by illumination at −5 °C was markedly suppressed (Figure 1, panel C).

When Ca^{2+} was added to treated membranes, the normal multiline signal was fully induced at −70 °C as well as at −5 and −40 °C (panel C) concomitant with appreciable suppression of the cyt *b*-559 signal, indicative of complementary relations between Mn oxidation and Fe oxidation. The restoration of the $g = 4.1$ signal, however, could not be confirmed because of incomplete subtraction of the sharp signal arising from rhombic iron and an unidentified signal co-induced at a similar magnetic field. The latter signal was observed in both low-pH-treated membranes and Ca^{2+}-repleted membranes independent of illumination temperature, but was not induced in untreated control membranes. This may suggest that Ca^{2+} depletion is not directly responsible for induction of this signal, although the origin of this signal is not clear.

Figure 2 shows the excitation temperature dependency of formation of the S_2 state as estimated from the amplitude of the EPR multiline signal. A 2-min dark incubation at 210 K was given before the illuminated samples were cooled to liquid He temperature to facilitate the possible transformation from the $g = 4.1$ state to the multiline state. In control untreated membranes, formation of S_2 became gradually inhibited below −80 °C, but a portion of the O_2 centers were still capable of S_2 formation even at −180 °C, in agreement with the result by Styring and Rutherford (1988). In low-pH-treated membranes, S_2 formation began to be affected at 0 °C and completely suppressed below −70 °C, indicating a marked up-shift by about 100 °C in the threshold temperature for S_2 formation. From the parallel translocation of the dependence curves, we may consider that the mechanism of Mn oxidation is not much changed, i.e., one-electron oxidation of the same Mn atom both before and after the treatment. On addition of Ca^{2+}, the temperature dependency was largely reversed to normal, although an appreciable shift toward higher temperatures by about 30 °C remained. This hysteresis suggests that some of the effects of low-pH treatment are irreversible. In fact, the $g = 4.1$ S_2 signal is not restored by Ca^{2+} addition.

Table I summarizes the effect of low-pH treatment and Ca^{2+} addition on the temperature dependency of the S_1 to S_2 transition as expressed by the half-inhibition temperature for formation of the multiline signal (present study) or thermoluminescence (Ono & Inoue, 1990a). In both measurements, the half-inhibition temperature for treated membranes was markedly up-shifted as compared to that for control untreated membranes. The up-shift was largely reversed when the treated membranes were supplemented with exogenous Ca^{2+}. The half-inhibition temperatures determined by the EPR multiline signal were significantly lower than those determined by thermoluminescence (Ono & Inoue, 1990a). This is probably due to the differences in illumination protocol:

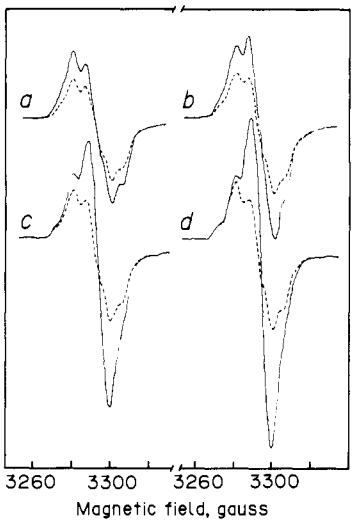

FIGURE 3: Effect of illumination temperature on EPR spectra of signals II_s and II_f. Samples were illuminated with continuous light at various temperatures: −5 °C (a), −30 °C (b), −35 °C (c), and −55 °C (d). Dashed curves indicate the spectra in darkness, and solid curves indicate the spectra during illumination. Sample concentration was 4 mg of Chl/mL. Instrumental settings: microwave power, 0.4 mW; microwave frequency, 9.26 GHz; modulation frequency and amplitude, 100 kHz and 4 G, respectively. Experimental details are described under Materials and Methods.

continuous illumination for EPR while a single-flash illumination for thermoluminescence. This may also be related to the observation that the transition efficiency decreases at low temperatures due to an increase in the miss factor.

Figure 3 shows signal II spectra at four different temperatures in low-pH-treated membranes. In this measurement, Y_D had been fully oxidized by preillumination as described under Materials and Methods. Signal II_f arising from Y_Z^+ was largely induced by illumination at −5 and −30 °C, supporting our previous conclusion that re-reduction of Y_Z^+ is suppressed in low-pH-treated membranes. Importantly also, signal II_f formation is totally suppressed by exogenous Ca^{2+} (Ono & Inoue, 1989a), indicating the Ca^{2+}-induced restoration of the low-pH effects. On further lowering the temperature, signal II_f formation became partially inhibited at −35 °C, concomitant with the appearance of a free radical signal probably due to the Chl cation, and only a very small signal II_f remained at −55 °C.

Figure 4 compares the dependency on excitation temperature of Y_Z^+ formation between low-pH-treated and NH_2OH-treated PSII. Y_Z^+ was estimated from the amplitude of light-induced signal II_f. In low-pH-treated PSII (closed circles), signal II_f became sharply inhibited below −30 °C followed by gradual inhibition below −50 °C. This temperature dependency is not characteristic of low-pH-treated PSII, since almost the same dependency curve was obtained for NH_2OH-treated PSII (open circles). The inhibition below −30 °C may be ascribed to the enhancement of charge recombination between Q_A^- and Y_Z^+ due to interruption of electron transfer from Q_A^- to Q_B below −30 °C (Joliot, 1974) or, alternatively, an increased probability of Chl oxidation as depicted in Figure 3. Note that this temperature dependency is largely different from that of the modified multiline formation (dashed curve), indicating that low-temperature-sensitive steps are not the same between Z oxidation and Mn oxidation.

The modified shape of the multiline signal shown in Figure 1 led us to assume that the Mn complex is structurally modulated after low-pH treatment. A way to collect information

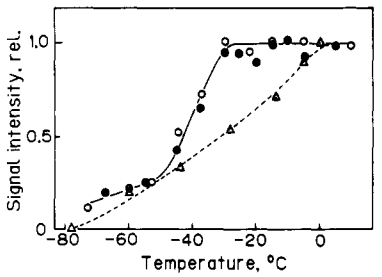

FIGURE 4: Temperature dependence of signal II_f formation in NH_2OH-treated PSII (○) and low-pH-treated PSII (●). Amplitudes of signals II_s and II_f were estimated from the peak heights and their light − dark differences, respectively, at 3278 G on the derivative spectra in Figure 3. Signal II_f amplitudes were normalized by relating to signal II_s amplitudes. The amplitude of the modified S_2 multiline signal in low-pH-treated PSII shown in Figure 2 was reproduced as a reference (△). Sample concentrations were 3 and 4 mg of Chl/mL for NH_2OH-treated PSII and low-pH-treated PSII, respectively. Conditions for EPR measurements were the same as in Figure 3. All signal amplitudes were expressed in percent relative to the maximum amplitudes obtained at high temperatures. Detailed conditions for measurement of the S_2 multiline signal were as described in Figure 1.

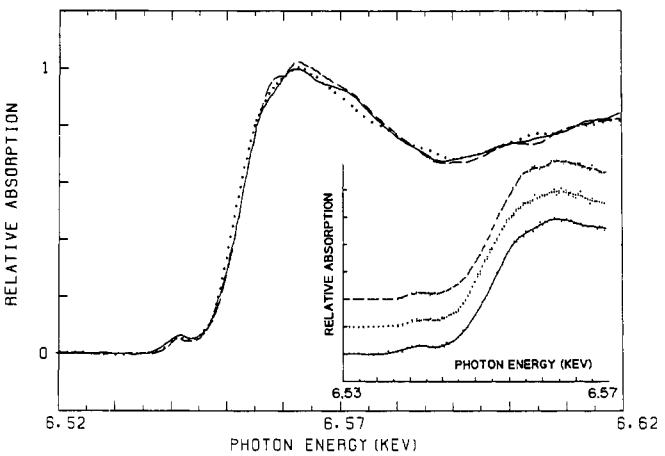

FIGURE 5: Effects of low-pH treatment on the Mn K-edge of XANES spectra; untreated control PSII (solid curve), low-pH-treated PSII (dotted curve), and low-pH-treated and then Ca^{2+}-repleted PSII (dashed curve). The spectra were processed by 9-point moving polynominal smoothing after subtraction of background counts. The same spectra with original data points are shown in the inset to indicate that the quality of measurement is good enough to resolve the slight shift (0.8 eV) of the K-edge energy induced by low-pH treatment.

about such a structural rearrangement of the Mn cluster is X-ray absorption spectroscopy. Figure 5 shows the XANES spectrum of Mn atoms in PSII membranes. The half-height energy of the Mn K-edge of untreated control membranes was located at 6552.0 ± 0.2 eV, whereas that of treated membranes was found at 6551.2 ± 0.2 eV, which was shifted to lower energy by 0.8 eV. On addition of Ca^{2+}, the half-height energy was completely reversed to that of untreated membranes (6552.0 ± 0.2 eV). The half-height energy of control membranes determined in this study was relatively higher (by 0.6 eV) as compared with that in our previous measurement (Kusunoki et al., 1990) and in some other literature (Goodin et al., 1984; Guiles et al., 1990; Penner-Hahn et al., 1990). At present, precise reasons for this difference are not clear. Generally, the energy of the K-edge inflection tends to be markedly affected by the energy-dependent fluctuation of background absorption or by intactness of the Mn cluster in the sample. We note, however, that the behavior of the K-edge upon low-pH treatment and Ca^{2+} addition was highly reproducible in three replicate experiments. It is inferred from these

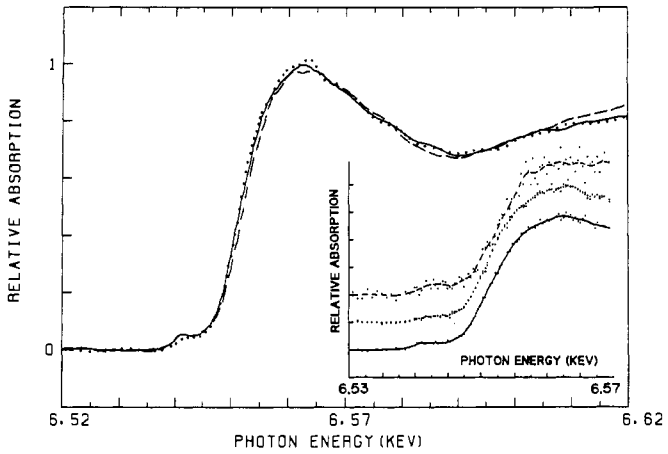

FIGURE 6: Effects of illumination on the Mn K-edge of XANES spectra in low-pH-treated PSII. Nonilluminated PSII (solid curve); PSII after illumination at 210 K for 15 min (dotted curve) and at 0 °C for 2.5 min (dashed curve). Dark-adapted PSII in the sample holder was illuminated from both sides with continuous light (white, 230 mW/cm^2). To minimize the heat effect, illumination was given intermittently (2.5-min light with 30-s dark intervals). Inset spectra with original data points were shown here for the same purpose as described in the Figure 5 legend.

results that the geometric and/or electronic structure of the Mn cluster is changed by the treatment, and Ca^{2+} addition reverses those changes to normal. Beside these reversible changes in half-height energy, the treatment also induced some additional changes in the profile of the principal absorption band of the spectrum, which were not always restored by the addition of Ca^{2+}. This suggests that the low-pH treatment involves not only the Ca^{2+}-reversible changes but also some irreversible structural modification of the Mn cluster. This seems to be in accordance with the facts that the $g = 4.1$ S_2 EPR signal is not restored by Ca^{2+} addition (Figure 1) and that the temperature dependence curve shows hysteresis upon Ca^{2+} addition (Figure 2). Such irreversible modifications, however, may not be so serious for the functioning of the Mn cluster, since O_2 evolution could be well restored by the addition of Ca^{2+}.

Figure 6 shows the effect of illumination on the Mn K-edge spectrum in low-pH-treated PSII. Illumination at 210 K is known to affect the S_1 to S_2 transition and induce an up-shift of the half-height energy by about 1 eV due to one-electron oxidation of Mn(III) to Mn(IV) (Yachandra et al., 1987). In treated PSII, however, illumination at 210 K was ineffective in inducing this up-shift, whereas illumination at 0 °C successfully induced a shift of the half-height energy from 6551.2 ± 0.2 to 6552.0 ± 0.3 eV. These results imply that in treated membranes, the Mn cluster is oxidized by illumination at 0 °C but not by 210 K illumination, which agrees with the observations by means of low-temperature EPR (Figure 1). The relatively small up-shift (0.8 eV) of the K-edge energy may reflect that some of the O_2 centers (20–30%) in treated membranes do not advance to the modified S_2 state (Ono & Inoue, 1990a). The good correspondence between the EPR data and XANES data with respect to the temperature dependence supports the view that the light-induced shift of the K-edge half-height energy is due to one-electron oxidation of the structurally modified Mn cluster in low-pH-treated O_2 centers.

Discussion

Present results showed that the temperature dependency of S_2 formation is markedly changed after low-pH treatment and that externally added Ca^{2+} restores the normal dependence. These results agree with our previous conclusion derived from thermoluminescence measurements (Ono & Inoue, 1990a). A simple interpretation for this phenomenon would be to assume that the temperature dependence of electron transport from Y_Z to $P680^+$ is modified by the treatment, referring to the fact that Ca^{2+} is required for this electron transport in cyanobacterial PSII (Satoh & Katoh, 1985). However, at a medium low temperature of −30 °C, signal II_f was fully photoinduced whereas the generation of modified multiline S_2 signal was already suppressed by 50% (Figure 4), indicating that the temperature dependences of these two EPR signals are dissimilar to each other. It is thus inferred that the low-temperature-sensitive site in low-pH-treated PSII does not reside in the electron transport from Y_Z to $P680^+$ but from Mn to Y_Z^+. Probably, the low-pH treatment gives rise to the abnormal temperature dependency by specifically modulating the electron abstraction from the Mn cluster in the S_1 state.

It has been reported that the S_1 to S_2 transition persists to lower temperatures (Styring & Rutherford, 1988) and has a relatively low activation energy as compared with the other S-state advances (Koike et al., 1987). Styring and Rutherford (1988) have interpreted this as related to the fact that all S-state transitions except S_1 to S_2 involve proton release; i.e., only a minimum conformational change is needed for the S_1 to S_2 transition. Extending their interpretation, one might consider that the up-shift in threshold temperature observed in this study implies the acquirement of a new function, the proton release coupled with the S_1 to S_2 transition in low-pH-treated PSII. This idea appears interesting in view of the data that the up-shifted temperature dependence roughly coincides with those of other S-state transitions. However, it does not seem to explain the other data that a positive charge was successfully accumulated in treated PSII as probed by a thermoluminescence band due to recombination between the negative charge on Q_A^- and the positive charge on the modified S_2 as previously reported (Ono & Inoue, 1990a). A more likely interpretation is that the Mn cluster in treated PSII is structurally modified to require more complicated conformational rearrangements on the S_1 to S_2 transition. As to the conformational rearrangements accompanying the S-state transitions, Styring and Rutherford (1988) have reported that little conformational change is coupled to the S_1 to S_2 transition, since the transition occurs at very low temperature. An opposite view is also possible: the S_1 to S_2 transition requires a larger conformational rearrangement to accommodate a positive charge. This idea is based on the observation that the activation entropy for the S_1 to S_2 transition is larger than that for the S_2 to S_3 transition (Koike et al., 1987). Whichever will be the case, ours and others results suggest that the putative structural modification of the Mn cluster brought about by the treatment disturbs the normal rearrangement of conformation.

The half-height energy of the Mn K-edge in the XANES spectrum was appreciably changed after low-pH treatment (Figure 5). This change may reflect modification of the Mn cluster due to Ca^{2+} extraction, since the change is reversed by the addition of Ca^{2+}. The shift of the K-edge toward lower energy (by 0.8 eV) can be interpreted in two ways: (1) One Mn atom in the tetranuclear cluster underwent a valence change from III to II, by analogy with the backward transition from S_1 to an S_0-like state reported to occur on incubation with NH_2OH followed by illumination (Guiles et al., 1990); (2) the ligation geometry of Mn atoms was modified, resulting in a less negative coordination environment. We may neglect the contribution by free Mn^{2+} from denatured O_2 centers, since

addition of Ca^{2+} could completely restore the normal K-edge half-height energy.

Interpretation 1 involves reduction of Mn. As to the Mn reduction, we may postulate two different mechanisms: (i) the Mn cluster in the normal S_1 state [Mn(III,III,III,III)] is spontaneously reduced at low pHs to form an S_0-like state [Mn(II,III,III,III)] by receiving one electron from an appropriate donor in the environment; (ii) a modified S_1 state [Mn(II,III,III,IV)] is generated as a result of either thermodynamically driven hydrolysis or disproportionation in the normal S_1 state, as proposed by Sivaraja et al. (1989). If (i) is the case, the expected product of one-electron oxidation will be either [Mn(III,III,III,III)] or [Mn(II,III,III,IV)], both of which do not account for the experimental fact that illumination of low-pH-treated PSII induced a modified EPR multiline signal. If (ii) is the case, the expected product of one-electron oxidation will be an S_2 state [Mn(II,III,IV,IV)] that is capable of exhibiting a modified multiline signal (Sivaraja et al., 1989). Notably, this modified S_2 state also explains the 0.8-eV up-shift of the K-edge energy as due to photooxidation of one Mn(III) to Mn(IV). On the basis of these mechanisms, we consider that mechanism ii is more likely. Presumably, the low-pH-induced removal of Ca gives rise to a charge imbalance in the Mn cluster to result in type ii valence redistribution.

Interpretation 2 assumes a low-pH-induced change in geometry of Mn ligation which results in a less negative coordination environment around the Mn cluster and thereby down-shifts the K-edge energy. Such changes in ligation geometry would result from a break of the coordination bond between the Mn cluster and coordinating ligands. We may presume that protonation of carboxyl residues will affect the coordination by analogy with the mechanism as proposed for Ca release on low-pH treatment that involves protonation of ligand residues (Ono & Inoue, 1988). In fact, Vermaas et al. (1990) have claimed the involvement of a carboxyl residue, Glu-69 in the D2 protein, in Mn binding. Apparently, we have to assume some role of Ca in this case either, since the changes are reversed by exogenous Ca^{2+}. Presumably, Ca^{2+} is required for optimization of the ligand position through stabilization of protein structure.

At present, we cannot present any unambiguous feature how the low-pH treatment or the consequent removal of Ca induces the down-shift of the K-edge energy. More detailed measurements of XANES spectra of low-pH-treated PSII particularly in the pre-edge region will provide new insights, since the high-energy end of the pre-edge absorption is a good indicator of the oxidation state of the Mn cluster (Kusunoki et al., 1990).

Shen et al. (1990) have reported that O_2 evolution by their PSII core preparation from a Chl *b* less rice mutant having only one Ca atom per PSII is inactivated by low-pH treatment without any loss in Ca abundance, but is reactivated by the addition of exogenous Ca^{2+}. On the basis of this finding, we have to interpret that Ca^{2+} loss is not the direct cause for modification of the Mn cluster. This idea, however, does not answer the question why Ca^{2+} is specifically required to restore the modified Mn cluster. At present, we have to reserve our final view as to the relation between Ca removal and (consequent) modification of the Mn cluster, since the number of Ca atoms per PSII is still a matter of debate: e.g., more than two Ca per PSII in spinach PSII core complex is reported by Kalosaka et al. (1990). Obviously, a more unambiguous determination of Ca abundance and its binding site is the key toward a thorough understanding of this phenomenon.

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