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Flash induced XANES spectroscopy for the Ca-depleted Mn-cluster in the photosynthetic O_2-evolving enzyme

Taka-aki Ono[a,*], Takumi Noguchi[a], Yorinao Inoue[a], Masami Kusunoki[b], Hirotaka Yamaguchi[c], Hiroyuki Oyanagi[a]

[a]*Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*
[b]*School of Science and Technology, Meiji University, Kawasaki, Kanagawa 214, Japan*
[c]*Electrotechnical Laboratory, Tsukuba, Ibaraki 305, Japan*

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Flash-induced changes of the Mn K-edge absorption spectra have been studied in the oxygen-evolving complex depleted of Ca. The Mn K-edge energy for the Ca-depleted S_1-state was lower by 1.5 eV than that for the normal S_1-state. The K-edge energy upshifted by 1 eV after one flash, indicative of an oxidation of Mn. After two flashes, the K-edge was elevated as well by 0.4 eV, and then reached a steady-state high level after continuous illumination where the K-edge energy was higher by 0.9 eV than that after one flash. The results indicate that the Mn-cluster and/or its direct ligand could be oxidized up to two electrons but further events are blocked.

O_2 evolution; Mn-cluster; Photosystem II; X-ray absorption; Calcium; EPR

1. INTRODUCTION

Ca is an indispensable metal cofactor for photosynthetic O_2 evolution, which takes place in the tetranuclear Mn-cluster (see [1–4] for reviews). The evidence for the location of the Ca atom in close vicinity of the Mn-cluster has been provided by means of X-ray absorption spectroscopy [5]. After treatment of the photosystem (PS) II membranes with citrate buffer at pH 3.0 (low-pH treatment), one Ca atom per PSII is selectively released concurrently with the loss of the capability of O_2 evolution which is restored by exogenously added Ca [6]. Illuminating the low-pH treated PSII membranes results in formation of a modified S_2-state as indicated by an altered multiline EPR signal [7–10], and an upshifted thermoluminescence band [8,11,12]. Further illumination of the abnormal S_2-state with a single flash or continuous illumination induces an EPR signal in the $g = 2$ region [7,9]. A quite similar EPR signal was also reported in the PSII membranes that were depleted of Ca by treatment with high concentrations of NaCl [13–16], and has been proposed to arise from the state where an oxidized radical of histidine [14] or tyrosine [16] interacts magnetically with the Mn-cluster, although the latter proposal has been challenged [17,18]. Recently we have succeeded in measuring XANES spectra of the Mn-cluster in dilute samples, which enable us to monitor the electronic state of the Mn-cluster in every intermediate S-state induced by saturating flash excitation under physiological conditions, and showed that the half-height energy of the Mn K-edge quadruply oscillates depending on flash number [19]. In the present study, we characterized the Mn-cluster in the modified intermediate states for water oxidation induced in the Ca-depleted OEC by means of flash induced XANES spectroscopy.

*Corresponding author. Fax: (81) (48) 462 4685.

Abbreviations PS, photosystem; OEC, oxygen evolving complex; XANES, X-ray absorption near edge structure, MES, 2-(*N*-morpholino)-ethanesulfonic acid.

2. MATERIALS AND METHODS

PS II membranes capable of O_2 evolution were prepared from spinach as described previously [11], and stored in liquid N_2 until use. After being thawed the membranes were relaxed by incubation for 6 h in the dark, and then subjected to low-pH treatment by incubation with 400 mM sucrose, 20 mM NaCl, 10 mM citrate-NaOH (pH 3.0) [6] with a modification as described in [12] in order to release a functional Ca. All the following procedures were done under dim-green safe light or in complete darkness, unless otherwise stated. The resulting membranes were washed once, and resuspended in 400 mM sucrose, 20 mM NaCl, 0.5 mM EDTA · 2Na, 40 mM MES-NaOH (pH 6.5) at a sample concentration of 5.7 mg chlorophyll/ml. A 18.0 μl aliquot of the sample was mounted on a membrane filter (8 mm × 5 mm) placed in a sample holder made of Cu, excited with a series of saturating Nd-YAG laser flashes (~7 ns, 20 mJ/cm^2, and 532 nm) at 10°C with an interval of 1 s between flashes or with continuous light at 0°C for 1 min by an incandescent lamp, and immediately cooled to 77 K in liquid N_2. This setup enables us to reduce the sample concentration that is low enough to ensure light saturation as described [19]. All these procedures were carried out within 60 min after low-pH treatment. XANES spectra were measured at the Photon Factory of the National Laboratory for High Energy Physics beam-line 4B under dedicated conditions (350–280 mA, 2.5 GeV) with a Si(111) crystal monochromator. Fluorescent X-ray was detected with a modified Si(Li) solid-

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state detector as described previously [19,20]. The sample holder was placed in a compact heat-insulation chamber as described [19] except that the detector was placed 1.0 cm from the sample. Samples were kept in darkness at 30K during data collection. Energy calibration was done by using the pre-edge peak at 6543.3 eV of the $KMNO_4$ spectrum measured before and after recording of the sample spectra. For each spectrum, ten scans were averaged. Processing of the original data points was carried out as described [19,20]

3. RESULTS AND DISCUSSION

Fig. 1A shows the processed XANES spectra of the Ca-depleted membranes. Data points presented in the processed spectra indicate that the quality of measurements is proper for resolving the half-height energy of the Mn K-edge, even though the sample concentration (ca. 120 μM Mn) was remarkably low. Fig. 1B shows the effect of the flash number on Mn K-edge spectra, in which five spectra are displayed on the same expanded energy scale to emphasize the shift of K-edge position after correction of the back-ground base line, which little affected the half-height energy of the Mn K-edge. The half-height energy in dark adapted membranes was located at 6550.2 eV. This K-edge energy was considerably lower than those reported in the normal S_1-state [20–22]. The result is consistent with our previous report using highly concentrated Ca-depleted PSII [10], where the K-edge energy completely reversed to that of untreated control membranes by the addition of Ca^{2+} concomitantly with the restoration of a high rate of O_2 evolution. The relatively small down-shift (0.8 eV) of the K-edge energy in our previous study may be partly attributable to the difference in sample preparation, although reasons for this difference are not clear at present. Similar changes in K-edge shape have been reported by Yachandra et al. [5]. The down-shift of the Mn K-edge induced by Ca-depletion can be ascribed either to the reduction of Mn or an alternation in the geometry of Mn ligation. From the findings that the normal K-edge is restored by Ca^{2+} addition in the dark [5,10], and that the modified S_1-state showed no characteristic EPR signal in a conventional detection mode [7–9,13–16], it was considered that the down-shift in the K-edge arises from a change in the ligation structure, e.g. a break of the coordination bond between Mn and coordinating ligand. The half-height energies of the spectra were determined to be 6551.2, 6551.6 and 6551.8 eV after 1, 2 and 3 flashes, respectively, and 6552.1 eV after continuous illumination. A quite similar K-edge up-shift by flash illumination was found in an independent experiment with a different batch of samples and machine time.

In Fig. 2, the half-height energy was plotted as a function of the flash number. No period-four flash pattern could be found as opposed to the normal OEC. Upon illuminating the dark-adapted membranes with a single turnover flash, the half-height energy was up-shifted by 1.0 eV. Since a single turnover of PSII in the

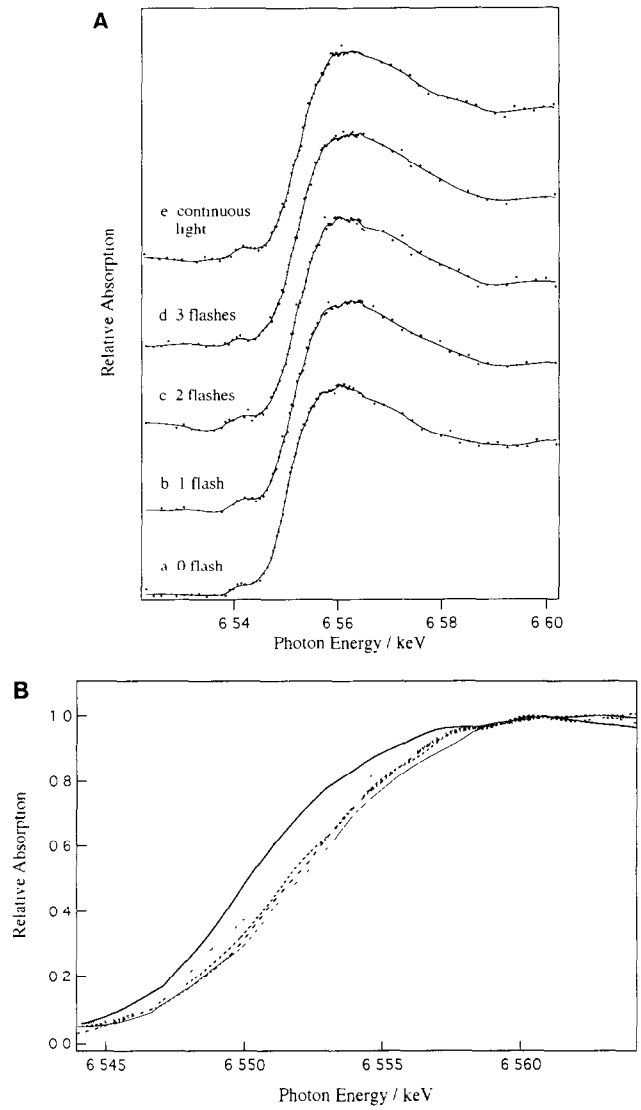

Fig. 1. (A) XANES spectra of Ca-depleted OEC after a series of flashes and continuous illumination. Spectra a, b, c, d and e correspond to 0, 1, 2 and 3 flashes, and continuous illumination, respectively. (B) Effect of illumination on the half-height energy of Mn K-edge. OEC after illumination with 0 (——), 1 (·····), 2 (– –), 3 (–·–) flashes and continuous illumination (———).

Ca-depleted OEC generates a modified multiline EPR signal [7–9,13–16], this up-shift of the K-edge is ascribed to the oxidation of Mn(III) to Mn(IV). After two flashes, the K-edge was elevated as well, but the extent of the up-shift was relatively smaller (0.4 eV). This indicates that the cycling of S-states is retarded after the formation of the modified S_2-state. After three flashes, the K-edge energy was again up-shifted by 0.2 eV to reach around 70% of a steady-state level found after continuous illumination where the K-edge energy was higher by 0.9 eV than that after one flash. Since illumination of the Ca-depleted S_2-state gives rise to the formation of a $g = 2$ EPR signal [7,9,13–18], we may conclude that the up-shift in Mn K-edge parallels the formation of the $g = 2$ EPR state. This view is furthermore

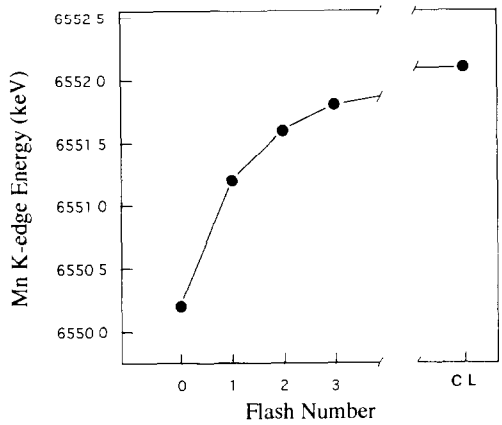

Fig. 2. Flash number dependent change of Mn K-edge energy of Ca-depleted OEC C.L. represents continuous illumination

supported by the fact that the flash pattern of the K-edge energy change subsequent to the first flash is quite similar to the reported flash pattern for the formation of the $g = 2$ EPR signal [9,14].

The present results provide evidence that the change in the electronic state of the Mn-cluster is directly involved in the formation of the $g = 2$ EPR state in the Ca-depleted OEC. Interestingly, the K-edge up-shift by 0.9 eV after continuous illumination coincides with that for the S_2-to-S_3 transition in native OEC [19]. The XANES spectroscopy with flash illumination can exclude the possibility that the found up-shift of the K-edge energy would be due to the product accumulating through some side-path reaction with low quantum efficiency. This may, therefore, suggest that the Ca-depleted PSII undergoes a chemistry similar to the native OEC upon the second flash illumination, and is consistent with the proposal that an advancement beyond the S_3-state is blocked in the absence of Ca [13,14]. A most probable interpretation for the K-edge up-shift upon formation of the $g = 2$ EPR state would be either an oxidation of Mn (III) atom as indicated in the S_1-to-S_2 transition, or an oxidation of its direct ligand upon formation of the $g = 2$ EPR state. This view seems to conflict with the proposal [14,16], that the $g = 2$ EPR signal arises from a rather weak exchange interaction between the Mn cluster and the organic radical, although we cannot completely exclude the possibility that some conformational rearrangement may be induced in the vicinity of the Mn-cluster by an oxidation of the protein residue upon formation of the $g = 2$ EPR state, resulting in a less negative coordination environment of the Mn-cluster. In a recent theoretical study [23], however, it has been shown that the $g = 2$ EPR signal could be simulated by assuming radical species generated by partial oxidation of substrate water to form the third bridging ligand between the di-μ-oxo Mn as a dimeric subunit in the Mn-cluster. This model predicts that the postulated water radical is EPR-silent in the normal S_3-state but becomes EPR detectable in the Mn-cluster under some special conformation, and may not contradict the up-shift of the K-edge energy. The K-edge energies of the Ca-depleted S_1-, S_2- and S_3-states were parallelly down-shifted compared with those of the respective S-states in the native OEC. The structural perturbation of the Mn-cluster in the Ca-depleted OEC as indicated by the down-shift of the K-edge energy may permit such kind of special arrangement of the Mn-cluster.

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