Drastic changes in the ligand structure of the oxygen-evolving Mn cluster upon Ca^{2+} depletion as revealed by FTIR difference spectroscopy

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Keywords: Ca^{2+}; carboxylate ligand; FTIR; Mn cluster; Oxygen evolution; Photosystem II

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FTIR, Fourier transform infrared; Mes, 2-(*N*-morpholino)ethanesulfonic acid; OEC, oxygen evolving center; PpBQ, phenyl-p-benzoquinone; PSII, photosystem II; Q_A, primary quinone electron acceptor.

Abstract

A Fourier transform infrared (FTIR) difference spectrum of the oxygen-evolving Mn cluster upon the S_1-to-S_2 transition was obtained with Ca^{2+}-depleted photosystem II (PSII) membranes to investigate the structural relevance of Ca^{2+} to the Mn cluster. Previously, Noguchi et al. [*Biochim. Biophys. Acta* 1228 (1995) 189] observed drastic changes in the carboxylate stretching region of the S_2/S_1 FTIR spectrum upon Ca^{2+} depletion, whereas Kimura and co-workers [*Biochemistry* 40 (2001) 14061; *ibid*. 41 (2002) 5844] later claimed that these changes were not ascribed to Ca^{2+} depletion itself but caused by the interaction of EDTA to the Mn cluster and/or binding of K^+ at the Ca^{2+} site. In the present study, the preparation of the Ca^{2+}-depleted PSII sample and its FTIR measurement were performed in the absence of EDTA and K^+. The obtained S_2/S_1 spectrum exhibited the loss of carboxylate bands at 1587/1562 and 1364/1403 cm^{-1} and diminished amide I intensities, which were identical to the previous observations in the presence of EDTA and K^+. This result indicates that the drastic FTIR changes are a pure effect of Ca^{2+} depletion, and provides solid evidence for the general view that Ca^{2+} is strongly coupled with the Mn cluster.

1. Introduction

Oxygen evolution in plants and cyanobacteria is performed at the oxygen-evolving center (OEC) in photosystem II (PSII) [1,2]. The chemical identity of OEC is the so-called Mn cluster, which consists of four Mn ions embedded in the protein matrix [3-5]. Molecular oxygen is evolved as a result of four-electron oxidation of two water molecules through a light-driven cycle of five intermediates called S states (S_0-S_4). By successive flash illumination, the dark stable S_1 state is transferred to the S_2, S_3, and S_0 states one after another, and returns back to the S_1 state. Molecular oxygen is released during the S_3-to-S_0 transition via the transient S_4 state.

Ca^{2+} has been known as an indispensable cofactor for oxygen evolution, and upon Ca^{2+} depletion, transitions beyond the S_2 state are blocked [1,6,7]. The recent X-ray crystal structures of the PSII core complexes of the cyanobactrium *Thermosynechococcus elongatus* at 3.5-3.0 Å resolutions [8,9] indeed showed that one Ca^{2+} ion is involved in the electron density of the Mn cluster. However, the details of the structural relevance of Ca^{2+} to the Mn cluster has not been revealed because of the relatively low resolutions of the X-ray structures [8,9] and possible damage to the Mn cluster by X-ray irradiation [10,11]. Several lines of evidence indicate that Ca^{2+} is not only a structural constituent of OEC but also directly involved in the chemical mechanism of oxygen evolution [7,12,13]. Thus, clarifying the structural relationship of Ca^{2+} to the Mn cluster and the role of Ca^{2+} in the reaction is crucial in understanding the whole mechanism of photosynthetic oxygen evolution.

Light-induced FTIR difference spectroscopy has been used as one of the powerful methods to study the detailed structures and reactions of OEC [14]. FTIR difference spectra upon S-state transitions [15,16] reveal the structural changes and reactions of amino

acid ligands [17-26], polypeptide chains [17-19], active water molecules [27,28], and the Mn cluster core [29,30]. In particular, the asymmetric and symmetric COO^- stretching vibrations of carboxylate groups show prominent bands in the mid-IR region of spectra, providing useful information to characterize the coordination structures of the carboxylate ligands to the Mn cluster [31-33].

Previously, Noguchi et al. [17] reported that upon Ca^{2+} depletion, the prominent COO^- peaks at 1560/1587 and 1403/1364 cm^{-1} in the S_2/S_1 difference spectra were lost in conjunction with the loss of intensity in the amide I bands of protein backbones. From this observation, it was proposed that there is a carboxylate ligand bridging Mn and Ca ions, which undergoes a drastic coordination change upon the S_2 formation concomitant with polypeptide backbone changes, and that upon Ca^{2+} depletion, this carboxylate ligand is released from the Mn ion [17]. Later, Kimura and co-workers [34-37] claimed in their studies using Chelex-treated buffers that Ca^{2+} depletion itself did not affect the carboxylate bands in the S_2/S_1 spectrum, but the presence of EDTA and/or K^+ caused the spectral changes via the interaction of EDTA with the Mn ion and/or binding of K^+ to the Ca^{2+} site. They also observed no appreciable changes in the low-frequency bands of the Mn-O-Mn core vibrations upon Ca^{2+} depletion [37]. However, their conclusion that Ca^{2+} depletion little affects the FTIR difference spectrum seems contradictory to the general view that Ca^{2+} is closely involved in the structure and reaction of the Mn cluster. In addition, the observation that Sr^{2+} substitution for Ca^{2+} clearly perturbed the S-state FTIR spectra [37-41], strongly suggesting that Ca^{2+} is structurally coupled to the Mn cluster, is consistent with the result by Noguchi et al. [17] but inconsistent with that by Kimura and co-workers [34-37]. Thus, the effect of Ca^{2+} depletion on the FTIR spectra of OEC is still

controversial and it is urgent to solve this problem for further FTIR investigation on the structural and functional role of Ca^{2+} in OEC.

In this study, we have reexamined the effect of Ca^{2+} depletion on the S_2/S_1 FTIR difference spectrum to resolve the discrepancy between the results of two groups. For this purpose, we have prepared the Ca^{2+}-depleted PSII membranes without using EDTA throughout the procedure. Instead, Chelex 100 was involved in the Ca^{2+}-depleted sample to prevent Ca^{2+} contamination during sample handling and even in FTIR measurement. In addition, the S_2/S_1 difference spectrum was obtained by taking a double difference between the Q_A^-/Q_A and $S_2Q_A^-/S_1Q_A$ spectra to avoid the presence of K^+ from potassium ferricyanide, which was used as an exogenous electron acceptor in the previous measurement [17]. Even in the absence of EDTA and K^+, the obtained S_2/S_1 spectrum was basically identical to the previous result by Noguchi et al. [17], showing drastic spectral changes in the carboxylate stretching and amide I regions. The result in the present study has provided solid evidence for the general view that Ca^{2+} is strongly coupled to the Mn cluster in the structure of OEC.

2. Materials and methods

The oxygen-evolving PSII membranes of spinach [42] were prepared as reported previously [43], and suspended in a pH 6.5 Mes buffer (Buffer A: 40 mM Mes, 400 mM sucrose, and 10 mM NaCl). Mn-depleted PSII membranes were prepared by 10 mM NH_2OH treatment to the sample suspension (0.5 mM Chl/ml). For the preparation of the control sample for FTIR measurement, the PSII suspension (5 mg Chl/ml) in 100 μl of Buffer A was diluted with 898 μl of water and then mixed with 2 μl of 5 mM DCMU/DMSO (final DCMU concentration: 0.01 mM). In the case of Mn-depleted

sample, the suspension was diluted with 888 µl of water and mixed with 10 µl of 100 mM NH_2OH (final NH_2OH concentration: 1 mM) in addition to 2 µl of DCMU/DMSO. The sample was then centrifuged at 7700 g for 5 min, and 880 µl of supernatant was removed. After suspension of the pellet in the remaining solution (120 µl), an aliquot of sample (10 µl) was loaded on a CaF_2 plate (25 mm in diameter) and dried under N_2 gas flow to make a dry film of PSII membranes. The sample was covered with another CaF_2 plate with a greased Teflon spacer (0.5 mm in thickness). In this sealed IR cell, 2 µl of 20 % (V/V) glycerol/water solution was placed without touching the sample to form a moderately hydrated film [44].

Ca^{2+} depletion was performed by low pH treatment [45,46]. The PSII membranes (3 mg Chl/ml) in a 0.1 mM Mes buffer (0.1 mM Mes, 400 mM sucrose, and 20 mM NaCl; pH 6.5) was added with the 1/3 volume of a pH 3.0 citrate buffer (40 mM citrate, 400 mM sucrose, and 20 mM NaCl) followed by incubation for 5 min on ice. Then, the 1/10 volume of a pH 7.5 Mops buffer (0.5 M Mops, 400 mM sucrose, and 20 mM NaCl) was added and the sample was incubated for 20 min on ice to rebind the 24 and 16 kDa extrinsic proteins. The Ca^{2+}-depleted PSII sample was centrifuged and the pellet was resuspended with Buffer A pretreated with Chelex 100 (Sigma) (Chelex-Buffer). Chelex 100 particles were further added to the sample suspension and the PSII membranes were washed four times with Chelex-Buffer. During this washing procedure, Chelex particles were always present in the sample suspension. The final precipitation (~0.5 mg Chl) by centrifugation was suspended in 100 µl of Chelex-Buffer, diluted with 898 µl of Milli-Q water, and then mixed with 2 µl of 5 mM DCMU/DMSO. The suspension without Chelex particles was transferred to another tube containing 1 mg of Chelex powder, which had been prepared by grinding in an agate mortar. The sample was centrifuged at 7700 g for 5 min and the 900

μl of supernatant was removed. The precipitation was suspended in the remaining solution and the aliquot of suspension (10 μl) containing both the Ca^{2+}-depleted PSII membranes and Chelex powder was loaded on a ZnSe plate. A hydrated film was then prepared in the same manner as the control sample. The ZnSe plates, glassware and tubes used in the preparation of the Ca^{2+}-depleted sample were rinsed with HCl solution prior to use.

For Ca^{2+} reconstitution, the Ca^{2+}-depleted PSII membranes (~0.5 mg Chl) were suspended in 1 ml of Buffer A in the presence of 20 mM $CaCl_2$, and incubated for one hour on ice. The sample was centrifuged and resuspended with the same buffer in 100 μl. The subsequent procedure to make a hydrated film in the presence of DCMU was the same as that for the control sample.

FTIR spectra were recorded using a Bruker IFS-66/S spectrophotometer equipped with an MCT detector (InfraRed D316/8) [44]. The sample temperature was adjusted to 10 ˚C by circulating cold water in a copper holder. Flash illumination was performed by a Q-switched Nd:YAG laser (Quanta-Ray GCR-130; wavelength, 532 nm; pulse width, ~7 ns fwhm; intensity, ~7 mJ $pulse^{-1}$ cm^{-2} at the sample surface). For $S_2Q_A^-/S_1Q_A$ measurements of control and Ca^{2+}-reconstituted samples, single-beam spectra (acquisition mode: double-sided fast return) with 10 scans (5-s accumulation) were recorded before and after single flash illumination. After dark relaxation for 12 min, the entire cycle was repeated 32 times, and spectra were averaged to calculate flash-induced $S_2Q_A^-/S_1Q_A$ difference spectra. For the Ca^{2+}-depleted sample, single-beam spectra with 80 scans (40-s accumulation) were recorded before and after a flash and a difference spectrum was calculated. Since the relaxation of the S_2 state in Ca^{2+}-depleted PSII is very slow [47], repetitive measurement using the same sample was avoided and four different samples were

used for measurements to average the spectra. A Q_A^-/Q_A spectrum was obtained using the Mn-depleted PSII membranes as a single-flash induced difference spectrum (100-s accumulation for each single-beam spectrum). Spectra of three different samples (no repetition for each sample) were averaged. The spectral resolution was 4 cm^{-1}.

Oxygen evolving activity was measured with a Clark-type oxygen electrode with PpBQ as an electron acceptor. For Ca^{2+}-depleted sample, Chelex 100 particles were involved in the PSII suspension during measurement. Upon Ca^{2+} depletion, oxygen evolving activity was lowered to 5% of that of the control sample (611 μM O_2 $mgChl^{-1}$ h^{-1}), and upon Ca^{2+} reconstitution, 63% of the activity relative to the control was recovered.

3. Results and discussion

Fig. 1 shows $S_2Q_A^-/S_1Q_A$ FTIR difference spectra (solid lines) of control (a), Ca^{2+}-depleted (b) and Ca^{2+}-reconstituted (c) PSII membranes together with a Q_A^-/Q_A difference spectra of Mn-depleted PSII membranes (dotted line). Throughout the procedures of the preparation of Ca^{2+}-depleted PSII and FTIR measurement, EDTA or an alternative chelator was not used. Although citrate, a weak chelator, was used in the low-pH treatment for Ca^{2+} depletion, its concentration in the final sample deposited on a ZnSe plate should be less than 1 nM after subsequent washing procedures. To avoid Ca^{2+} contamination, instead of using soluble chelators, Chelex 100 particles were always present in the sample suspension and also the powder of Chelex 100 was loaded together with the PSII sample to make a hydrated film. A hydrated film as a sample form has an advantage in the spectral measurement in the presence of Chelex powder, because making a thin pellet sample (<10 μm in thickness) between IR windows [17] is difficult with grains of Chelex. An electron flow was blocked at Q_A by the presence of DCMU. Thus, potassium

ferricyanide as an exogenous electron acceptor was not necessary, and hence K^+, which was asserted to interact with the Ca^{2+} binding site [35], was not involved in the sample.

The control sample in a hydrated film (Fig. 1a, solid line) showed a typical $S_2Q_A^-/S_1Q_A$ spectrum of PSII membranes [34,48], and the Q_A^-/Q_A spectrum of the Mn-depleted sample (Fig. 1, dotted lines) was basically identical to the previous spectra [49,50]. The prominent peak at 1477 cm^{-1} is ascribed to the CO stretching vibration of Q_A^- [49], and peaks from the OEC were observed at 1678(-), 1650(+),1586(+), 1561(-), 1502(+), 1420(-), 1403(-), and 1364(+) cm^{-1} [17]. The $S_2Q_A^-/S_1Q_A$ spectrum after Ca^{2+} reconstitution (Fig. 1c, solid line) was very similar to the control spectrum. By contrast, the Ca^{2+}-depleted PSII sample showed rather different spectral features (Fig. 1b, solid line). The peak intensities at 1678, 1650, 1586, 1561, 1403, and 1364 cm^{-1} clearly decreased while those at 1502 and 1420 cm^{-1} remained.

To reveal the effects of Ca^{2+} depletion on the FTIR spectrum more clearly, the S_2/S_1 double difference spectra were calculated by subtracting the Q_A^-/Q_A spectrum from the $S_2Q_A^-/S_1Q_A$ spectra (Fig. 2). The S_2/S_1 spectrum of the control sample (Fig. 2a) was virtually identical to the previous S_2/S_1 spectrum measured as a single flash-induced difference spectrum in the presence of an exogenous electron acceptor, ferricyanide [17]. The prominent peaks at 1435(+)/1420(-)/1403(-)/1364(+) cm^{-1} have been assigned to the symmetric COO^- stretching vibrations of carboxylate groups that are probably the ligands to the Mn cluster, while the peaks at 1587(+)/1562(-)/1552(+)/1544(-)/1531(+)/1522(-)/1501(+) cm^{-1} have been ascribed to the asymmetric COO^- stretching vibration or to the amide II bands of protein backbones [17,19]. The sharp peaks in the 1700-1600 cm^{-1} region arise from the amide I vibrations of protein backbones, and the appearance of these peaks together with the amide II bands

indicates that there are drastic changes in the structures of polypepide chains around the Mn cluster upon the S_1-to-S_2 transition.

The S_2/S_1 double spectrum of the Ca^{2+}-depleted sample (Fig. 2b) showed totally different spectral features from that of the control sample. First, in the symmetric COO^- region, the strong peaks at 1403/1364 cm^{-1} were lost, while the neighbouring medium peaks at 1435/1420 cm^{-1} remained with a slight shift to the lower frequency by 2-3 cm^{-1}. Second, in the asymmetric COO^- and amide II region, the large band at 1587 cm^{-1} was lost and the intensity of the negative band at 1562 cm^{-1} significantly decreased, while the positive peak at 1501 cm^{-1} was basically unchanged in intensity although the peak frequency slightly upshifted. Third, most of the intensities in the amide I bands were lost, indicating that the structural changes in the polypeptide chains were largely restricted in Ca^{2+}-depleted PSII. Upon Ca^{2+} reconstitution (Fig. 2c), the above bands were mostly recovered, indicating that the spectral changes upon Ca^{2+} depletion was not attributed to the impairment of the Mn cluster. The reason for the somewhat weaker intensities in the amide I bands in the Ca^{2+}-reconstituted spectrum (Fig. 2c) relative to those in the control spectrum could be caused by partial release of extrinsic proteins during the Ca^{2+} depletion procedure or an unidentified perturbation by low pH treatment [51]

The above changes in the S_2/S_1 spectrum upon Ca^{2+} depletion are basically identical to our previous result of the S_2/S_1 spectrum of Ca^{2+}-depleted PSII membranes [17]. In the latter study, 0.5 mM EDTA was involved in the sample to prevent Ca^{2+} contamination, and also K^+ ions were present because potassium ferricyanide was used as an exogenous electron acceptor. The presence of chelators and K^+ was claimed to cause the spectral changes in Ca^{2+}-depleted PSII by Kimura and co-workers [34-37]. In contrast, in the measurements of the present study, neither soluble chelators nor K^+ ions are involved

in the Ca^{2+}-depleted PSII sample. Note that the citrate contaminant (<1 nM) cannot be responsible for the loss of the COO^- bands because it was reported that citrate showed only a weak effect on the COO^- bands and most of the COO^- intensities remained even in the presence of 5 mM citrate [36]. Therefore, the present experiment definitely demonstrates that Ca^{2+} depletion itself gives rise to the above drastic changes in the S_2/S_1 difference spectrum, and these changes are not derived from the effects of chelators and K^+ ions.

Kimura and co-workers [34-37] claimed that Ca^{2+} depletion did not affect the S_2/S_1 difference spectrum in the carboxylate stretching region and even in the low-frequency region of the Mn cluster core vibration. Spectra were changed only when soluble chelators and metal ions that can substitute for the Ca^{2+} ion are present in samples. Although they proposed that the interaction of a chelator to the Mn cluster changed the ligand structure by replacement of a native carboxylate ligand, no chelator bands were identified in the spectra [34,36]. Thus, it is highly likely that the unchanged FTIR spectrum was attributed to the contamination of Ca^{2+} in their "Ca^{2+}-depleted" samples and the presence of soluble chelators or metal substitution was necessary to remove Ca^{2+} from its binding site. They adopted a NaCl wash as a method for Ca^{2+} depletion. The treatment with high concentration NaCl (2 M) would provide a higher chance of contamination of Ca^{2+}. In addition, this treatment removes the 23 and 17 kDa extrinsic proteins from the PSII complex. The absence of these extrinsic proteins may facilitate the access of contaminating Ca^{2+} to the Mn cluster. In fact, it is known that the rate of binding of Ca^{2+} to the Ca^{2+}-depleted PSII in the absence of the two extrinsic proteins is significantly higher than to the sample in the presence of these proteins [52].

The results in the present study indicate that drastic changes take place in the ligand structure of the Mn cluster upon Ca^{2+} depletion. This conclusion is consistent with

the results of the EXAFS [3] and X-ray crystallographic [8,9] studies, in which the Ca^{2+} ion exists in the close vicinity of the Mn cluster and is connected to the Mn ions via μ-oxo and/or carboxylate bridges. Also, this conclusion is consistent with the previous FTIR results that upon Sr^{2+} substitution for Ca^{2+}, the S-state FTIR spectra were perturbed in the symmetric and asymmetric COO^- stretching regions [37-41] and also in the low-frequency region of the Mn-O-Mn core vibration [30,37], which are indicative of the rearrangement of the ligand and core structure of the Mn cluster by insertion of Sr^{2+} having a larger ionic radius than Ca^{2+}.

In the previous report [17], we proposed from the observation of the loss of the COO^- bands at 1587/1562 and 1403/1364 cm^{-1} upon Ca^{2+} depletion that a certain carboxylate ligand closely coupled to the Ca^{2+} ion undergoes a drastic coordination change in the S_1-to-S_2 transition, and upon Ca^{2+} depletion this ligand is released from the Mn cluster. From the general correlation between the coordination structure of a carboxylate ligand and the frequency gap between the asymmetric and symmetric vibrations [31-33], we further proposed, as a most straightforward interpretation, that there is a bridging carboxylate connecting Mn and Ca ions in the S_1 state and the coordination to Ca is broken upon the S_2 formation. This drastic coordination change can be coupled to the large structural changes in the polypeptide chains represented by the presence of strong amide I bands (Fig. 2a). Upon release of this carboxylate group from the Mn cluster, the polypeptide changes are also not induced anymore, resulting in the diminished intensity of the amide I bands (Fig. 2b) [17]. It is possible that these ligand and polypeptide changes are a prerequisite for the next S_2-to-S_3 transition, which is blocked in Ca^{2+}-depleted PSII.

According to the model of the Mn cluster from the 3Å X-ray structure of PSII by Loll et al. [9], Ca^{2+} is connected to the Mn ions through two bridging carboxylate ligands

from the α-COO^- of D1-Ala344 and the side chain of D1-Glu189. However, the symmetric stretching band of α-COO^- of D1-Ala344 has been assigned to ~1356 cm^{-1} in the S_1 state and to ~1337 or ~1320 cm^{-1} in the S_2 state by L-[1-^{13}C]alanine labelled core complexes of *Synechocystis* sp. PCC6803 [20]. In addition, the recent careful study by Strickler et al. [22] using site-directed mutants at D1-Glu189 showed that the COO^- bands of this carboxylate group little contribute to the FTIR spectra during the S-state cycle. Thus, at least the symmetric carboxylate bands at 1403/1364 cm^{-1} that are lost upon Ca^{2+} depletion are ascribed to neither D1-Ala344 nor D1-Glu189. Hence, the above view by the straightforward interpretation of FTIR data is not consistent with this Mn-cluster model by X-ray crystallography. It could be possible that the concerned carboxylate ligand does not bridge the Ca and Mn ions but bridges two Mn ions, and for some reason the coordination change of this carboxylate ligand upon S_2 formation takes place only when the Ca^{2+} ion is present in the vicinity. Carboxylate groups of CP43-Glu354, D1-Asp342, and D1-Glu333, which form bridging ligands to the Mn ions in the X-ray model [9], are possible candidates in this scheme. In contrast, it has been proposed that the X-ray crystallographic data represent the structures of the Mn ions in uncontrolled low redox states due to X-ray damage [10,11]. In this case, our model of the coordination change of the carboxylate bridge between the Ca and Mn ions could remain as a possible mechanism of the role of Ca^{2+} in photosynthetic oxygen evolution.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research (17GS0314 and 18570145) from the Ministry of Education, Science, Sports, Culture and Technology, and by Special Research Project "NanoScience" at the University of Tsukuba.

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Figure Captions

Fig. 1. $S_2Q_A^-/S_1Q_A$ FTIR difference spectra (solid lines) of control (a), Ca^{2+}-depleted (b), and Ca^{2+}-reconstituted (c) PSII membranes. A Q_A^-/Q_A difference spectrum of Mn-depleted PSII membranes (dotted lines) is shown in each panel for comparison. The spectra were normalized to the CO peak of Q_A^- at 1477 cm^{-1}. Samples as hydrated films in the presence of DCMU were illuminated by a single flash from a Nd:YAG laser (532 nm) and difference spectra were recorded. The sample temperature was adjusted to 10 °C.

Fig. 2. S_2/S_1 FTIR difference spectra of control (a), Ca^{2+}-depleted (b), and Ca^{2+}-reconstituted (c) PSII membranes. Spectra were obtained by subtracting the Q_A^-/Q_A spectrum from the $S_2Q_A^-/S_1Q_A$ difference spectra of individual samples shown in Fig. 1.