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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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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.

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

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 cyanobacterium *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 have 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

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

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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 1 h 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.