Structural Changes of D1 C-terminal α-Carboxylate during S-state Cycling in Photosynthetic Oxygen Evolution*

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Changes in the chemical structure of α-carboxylate of the D1 C-terminal Ala-344 during S-state cycling of photosynthetic oxygen-evolving complex were selectively measured using light-induced Fourier transform infrared (FTIR) difference spectroscopy in combination with specific [1-13C]-alanine labeling and site-directed mutagenesis in photosystem II core particles from Synechocystis sp. PCC 6803. Several bands for carboxylate symmetric stretching modes in an S1/S0 FTIR difference spectrum were affected by selective [13C]-labeling of the α-carboxylate of Ala with L-[1-13C]-alanine, whereas most of the isotopic effects failed to be induced in a site-directed mutant in which Ala-344 was replaced with Gly. Labeling of the α-methyl of Ala with L-[3-13C]-alanine had much smaller effects on the spectrum to induce isotopic bands due to a symmetric CH2 deformation coupled with the α-carboxylate. The isotopic bands for the α-carboxylate of Ala-344 showed characteristic changes during S-state cycling. The bands appeared prominently upon the S1-to-S2 transition and to a lesser extent upon the S2-to-S3 transition but reappeared at slightly upshifted frequencies with the opposite sign upon the S3-to-S0 transition. No obvious isotopic band appeared upon the S0-to-S1 transition. These results indicate that the α-carboxylate of C-terminal Ala-344 is structurally associated with a manganese ion that becomes oxidized upon the S1-to-S2 transition and reduced reversely upon the S3-to-S0 transition but is not associated with manganese ion(s) oxidized during the S2-to-S1 (and S3-to-S0) transition(s). Consistently, L-[1-13C]-alanine labeling also induced spectral changes in the low frequency (670–350 cm\(^{-1}\)) S2/S1 FTIR difference spectrum.

Photosynthetic water oxidation takes place in an oxygen-evolving complex (OEC)\(^1\) in which the catalytic metal cluster located on the luminal side of the D1 protein is composed of four manganese ions and one Ca\(^{2+}\) ion. Most of the potential ligands of the manganese/Ca\(^{2+}\) cluster appear to be located on the D1 protein based on site-directed mutagenesis studies using the cyanobacterium Synechocystis sp. PCC 6803 (reviewed in Refs. 1–3). These include Asp-170, Glu-189, His-190, His-332, Glu-333, His-337, Asp-342, and C-terminal Ala-344 (4–9), which are arranged in close proximity to the cluster according to the x-ray structural model of S1 state OEC (10–13).

The D1 protein is synthesized and assembled into the PS II complex with a short C-terminal extension except for the protein in Euglena (14). Light-dependent assembly of the manganese/Ca\(^{2+}\) cluster requires a free α-carboxylate of the C-terminal Ala-344, which occurs via cleavage of the C-terminal extension by the D1 C-terminal processing protease (CtpA) (15). None of the C-terminal truncated Synechocystis mutants in which Asn-335, Asp-342, Leu-343, and Ala-344 were replaced with a stop codon grew photoautotrophically and evolved oxygen (5). Site-directed replacement of D1-Ala-344 with Gly, Met, Ser, Val, Glu, or Gln in the D1-Ala-344-stop strain did not eliminate the capability for photoautotrophic growth and oxygen evolution (5). Light-induced FTIR difference spectroscopy showed the isotope-induced changes of carboxylate symmetric stretching bands from the α-carboxylate of Ala-344 upon the S1-to-S2 transition by incorporating L-[1-13C]-alanine isotope (16). Replacement of the C-terminal Ala with Gly, which induced marked changes in the skeletal structure of the manganese/Ca\(^{2+}\) cluster detected by light-induced FTIR difference spectroscopy in the low frequency region (17), indicated the close structural association of Ala-344 with the manganese/Ca\(^{2+}\) cluster. These observations suggest that the α-carboxylate of D1-Ala-344 associates with manganese ion(s) as a direct ligand. Some x-ray structural models of PS II suggested possible ligation of the α-carboxylate of Ala-344 to the manganese ion(s) (10–12), but a recent 3.5 Å x-ray model proposed the closest location of the α-carboxylate to the Ca\(^{2+}\) ion that composes a cubane-like cluster core with three manganese ions (13). Notably, however, no electron density for the α-carboxylate of Ala-344 has been resolved in any x-ray models because of limited resolution and x-ray damages during data collection.

During the process of photosynthetic oxygen evolution, two water molecules are oxidized to yield an oxygen molecule through five intermediates labeled S\(_n\) (n = 0–4), where n denotes the number of oxidizing equivalents stored. Each S\(_n\) state advances to the S\(_{n+1}\) state by absorbing a photon until reaching the highest oxidation state, S4, which spontaneously reverts to the lowest oxidation state, S0, concomitant with the release of an oxygen molecule (18, 19). Because the thermally stable S1 state predominates after prolonged dark incubation, S-state cycling starts from the S1 state in the dark adapted OEC. However, the mechanism through which substrate water molecules are oxidized to an oxygen molecule remains still largely unknown. To understand the mechanism of water oxidation, it is indispensable to know the details of changes in the chemical structure of the OEC during S-state cycling. Infrared

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‡ The abbreviations used are: OEC, oxygen-evolving complex; FTIR, Fourier transform infrared; Mes, 2-morpholinoethanesulfonic acid; PS, photosystem.
spectroscopy is a powerful technique for detecting subtle changes in molecular structure and chemical bonds accompanying chemical processes directly (20). Mid-frequency FTIR difference spectroscopy has been applied to studies on oxygen evolution for elucidating S-state-dependent changes in protein backbone and amino acid side groups, which structurally associate with the manganese/Ca$^{2+}$ cluster in a direct or indirect manner (21–25). However, the spectra obtained were a composite of changes of a large number of bands, and no band was exactly assigned to a specific amino acid residue in a protein, with the only exception being the bands for the α-carboxylate of the C-terminal Ala-344 of the D1 protein found in the $S_2/S_1$ FTIR difference spectrum (16).

In the present study, we characterized the bands of C-terminal Ala-344 of the D1 protein in the mid-frequency $S_2/S_1$ FTIR difference spectrum by labeling the α-carboxylate group with L-[1-13C]alanine and the α-methyl group with L-[3-13C]alanine in combination with site-directed mutation of Ala-344. We further report the changes in the mid-frequency bands for the α-carboxylate of C-terminal Ala-344 during S-state cycling in PS II core particles from Synechocystis sp. PCC 6803. This is the first report on detection of the S-state-dependent structural changes in a single amino acid ligand for the manganese/Ca$^{2+}$ cluster in OEC. It was also shown that bands in the low frequency (670–350 cm$^{-1}$) $S_2/S_1$ FTIR difference spectrum exhibited characteristic changes due to L-[1-13C]alanine labeling.

**EXPERIMENTAL PROCEDURES**

*Sample Materials—* A mutant with Gly instead of Ala at the D1 C-terminal (A344G-stop) was constructed based on the control strain of *Synechocystis* sp. PCC 6803, which lacks the D1 C-terminal extension and bears a His tag on the C-terminal of CP47 (Ala-344-stop) (17, 26). For specific 13C labeling of the α-carboxylate (1-C) or α-methyl carbon (3-C) of alanine, cells were grown photoautotrophically in BGL1 medium supplemented with 0.5 mM l-[1-13C]alanine or l-[3-13C]alanine (99% 13C enrichment, Cambridge Isotope Laboratories Inc.). Total incorporation of 13C into 1-C or 3-C of Ala was evaluated with liquid chromatography/mass spectrometry analysis and found to be ~70% in thylakoid membranes. Thylakoid membranes obtained by disrupting cells using a Bead-Beater (Bio-Spec Products) followed by centrifugation were solubilized with n-dodecyl-β-D-maltoside, and PS II core particles were chromatographically purified using a nickel column (17, 26). The sample medium was replaced with 40 mM sucrose, 5 mM NaCl, 5 mM CaCl$_2$, 10 mM Mes/NaOH, pH 6.0 (medium A) by precipitation of PS II core particles using 10% (w/v) polyethylene glycol 6000 and extensive washes with medium A for $S_2/S_1$ FTIR difference spectra (17) or repeated ultrafiltration in the presence of 0.06% (w/v) n-dodecyl-β-D-maltoside for S-state cycling (25). The O$_2$-evolving activity of the core particles obtained was ~2500 μmol of O$_2$ (mg of chlorophyll)$^{-1}$ h$^{-1}$ at 25 °C using 4 mM potassium ferricyanide as an electron acceptor.

**FTIR Measurements—** Mid-frequency (1800–1200 cm$^{-1}$) and low frequency (670–350 cm$^{-1}$) FTIR spectra were recorded on a Bruker IFS-66v/S spectrophotometer equipped with a mercury cadmium telluride detector (EG&G Optoelectronics D316/6) and on a Bomen MB102 spectrophotometer equipped with a silicon bolometer (infrared, HDL-5), respectively, at 0 °C (±0.03 °C) as described previously (25, 26). The PS II core particles (~40 μg of chlorophyll) suspended in medium A were mixed with 1 μl of sodium ferricyanide solution (100 mM stock) as an electron acceptor. The PS II core suspension was deposited on a BaF$_2$ or AgCl disk with a greased Teflon spacer after placing a droplet of 20% (v/v) glycerol/water solution adjacent to the sample for rehydration (25). After dark incubation at 0 °C for 1 h, the sample was preflashed to reduce the oxidized non-heme iron on the acceptor side and to enrich the S$_0$ population. The sample was subjected to one flash for the S$_1$-to-S$_2$ transition or four successive flashes at 10-s intervals for S-state transitions provided from a frequency-doubled Nd$^{3+}$:YAG laser (Spectra Physics, INDI-50) as described previously (25, 26). Single-beam spectra in the mid-frequency (20 scans) or low frequency (10 scans) region were measured before and after the first flash for $S_0/S_1$ difference or before the first flash and after each flash for the measurement of the flash-induced difference spectrum of each transition during the S-state cycling. To obtain difference spectra for the respective S-state transitions, the single-beam spectrum before each flash was subtracted from that after the flash. To obtain higher quality spectra of $S_0/S_1$ difference for close inspection of the isotopic bands than the spectra for S-state cycling, 80–170 and 70–89 mid-frequency spectra were accumulated for $S_0/S_1$ difference and S-state cycling, respectively. 261–273 spectra were averaged for the low frequency region.

**RESULTS**

*Effects of $^{13}$C-Alanine Labeling on $S_0/S_1$ FTIR Difference Spectrum—* Fig. 1A shows mid-frequency $S_0/S_1$ FTIR difference spectra of PS II core particles from the control Ala-344-stop cells labeled by L-[1-13C]alanine (a, red line) or L-[3-13C]alanine (b, red line) and the particles from A344G-stop cells labeled by L-[1-13C]alanine (c, red line) as compared with the unlabeled spectra (a–c, black lines). Carbon atoms of the α-carboxylate and α-methyl groups in the alanine residues were selectively labeled by L-[1-13C]alanine and L-[3-13C]alanine, respectively. The $S_0/S_1$ spectra include the symmetric (1450–1300 cm$^{-1}$) and asymmetric (1600–1500 cm$^{-1}$) stretching modes from putative carboxylate ligands for the manganese/Ca$^{2+}$ cluster as
well as the amide I (1700–1600 cm\(^{-1}\)) and II (1600–1500 cm\(^{-1}\)) modes from polypeptide backbones (16, 24–26). Labeling by \(\text{L-}[1-^{13}\text{C}]\)alanine induced changes of \(S_2/S_1\) spectra in the carboxylate symmetric stretching region below 1400 cm\(^{-1}\) in particles from the control Ala-344-stop cells (a) but minimal changes in particles from the mutant A344G-stop cells (c). These results were in good agreement with a previous report (16) and indicated that changes in the \(\alpha\)-carboxylate group of D1 C-terminal Ala-344 are mainly responsible for these isotopic bands. Consistent with this view, labeling of the \(\alpha\)-methyl of alanine by \(\text{L-}[3-^{13}\text{C}]\)alanine had little influence on spectra in the carboxylate symmetric stretching region of the control Ala-344-stop particles (b) other than the slight difference around 1354 cm\(^{-1}\). Although these isotope labeling experiments also induced changes in the spectra at 1700–1500 cm\(^{-1}\), the region that includes asymmetric stretching bands of carboxylates, the effects of the isotope labeling on the spectra in this frequency region were ambiguous at present due to extensive overlap of intense amide bands.

Fig. 1B shows double difference FTIR spectra for the \(S_2/S_1\) difference in the carboxylate symmetric stretching region (1500–1200 cm\(^{-1}\)), in which relatively small changes induced by the isotope labeling were clearly observable due to the absence of overlapping by intensive amide I and II bands. Each double difference spectrum was obtained by subtracting the \([1-^{13}\text{C}]\)alanine-labeled spectrum from the unlabeled \([1^{12}\text{C}]\)alanine spectrum \((1500–1200\text{ cm}^{-1})\). In contrast, the \([1^{13}\text{C}]\)alanine spectrum \((1800–1200\text{ cm}^{-1})\) shows double difference FTIR spectra for the \(S_2/S_1\) difference in the carboxylate symmetric stretching region (1500–1200 cm\(^{-1}\)), in which relatively small changes induced by the isotope labeling were clearly observable due to the absence of overlapping by intensive amide I and II bands. Each double difference spectrum was obtained by subtracting the \([1^{13}\text{C}]\)alanine-labeled spectrum from the unlabeled \([1^{12}\text{C}]\)alanine spectrum \((1500–1200\text{ cm}^{-1})\).

The obtained difference spectrum was very similar to that previously reported at 1375–1290 cm\(^{-1}\) (16). The positive bands can be ascribed to \([-1^{13}\text{C}]\)Ala-344 in the \(S_2\) state or \([1-^{13}\text{C}]\)Ala-344 in the \(S_1\) state, and the negative bands can be ascribed to \([-1^{13}\text{C}]\)Ala-344 in the \(S_1\) state or \([1-^{13}\text{C}]\)Ala-344 in the \(S_2\) state. Most of the bands observed in the \([-1^{13}\text{C}]\)Ala-344 spectra for the control Ala-344-stop particles were not present in the \([1^{13}\text{C}]\)Ala-344 spectra for the control Ala-344-stop particles (a). However, derivative-dense bands were induced by the \(^{13}\text{C}\) labeling of \(\alpha\)-methyl in alanine. The symmetric CH\(_2\) deformation of \(\alpha\)-methyl and the symmetric COO\(^{-}\) stretching modes of \(\alpha\)-carboxylate in alanine were coupled (27), and the coupled bands were affected by the \(^{13}\text{C}\) (16) or \(3^{11}\text{C}\) labeling in the \(12^{15}\text{C}\) difference spectrum of alanine solution. In contrast, the asymmetric CH\(_2\) deformation mode was hardly affected by both types of labeling, and the CH bending mode sensitive to the \(1^{13}\text{C}\) labeling was little changed upon the \(3^{11}\text{C}\) labeling. Therefore, the 1536\((\pm)\)1349\((\pm)\) cm\(^{-1}\) bands in the \([3^{12}\text{C}]/[3^{13}\text{C}]\)alanine spectrum (b) or in the \([-1^{13}\text{C}]\)Ala-344 spectrum for the control Ala-344-stop particles (c) and S0/S3 (c) differences spectra induced upon the first, second, third, and fourth flash illumination, respectively, are shown.

Structural Changes of D1 C-terminal Ala-344 Carboxylate during S-state Cycling—Fig. 2 shows the FTIR difference spectra at 1800–1200 cm\(^{-1}\) obtained during S-state cycling of PS II core particles from the unlabeled control (black lines) and \([-1^{13}\text{C}]\)alanine-labeled (red lines) cells. The difference spectra induced by the first (a), second (b), third (c), and fourth (d) flashes correspond to the \(S_2/S_1\), \(S_3/S_2\), \(S_0/S_3\), and \(S_2/S_0\) difference spectra, respectively. Spectra showed characteristic changes that were dependent on the number of flashes. Each spectrum for the \(S_2\)-state cycling of unlabeled control (Ala-344-stop) particles was nearly identical to that of particles isolated from the histidine-tagged wild-type Synechocystis strain retaining the C-terminal extension (25) and very similar to PS II core particles from Thermosynechococcus elongatus (21, 23, 24) and spinach (22, 25). Overall features of the \([-1^{13}\text{C}]\)alanine-labeled spectra for \(S\)-state cycling were similar to those for the unlabeled control, but distinct changes were clearly observed in the carboxylate symmetric stretching regions below 1400 cm\(^{-1}\) in spectra induced by the first (a) and third (c) flashes but not in those induced by the second (b) and fourth (d) flashes. Spectral features in the 1700–1500 cm\(^{-1}\) region showed some differences in band intensity between the unlabeled controls and \([-1^{13}\text{C}]\)alanine-labeled spectra. This frequency region is expected to involve asymmetric carboxylate stretching vibrations that are the counterpart of the labeling-sensitive symmetric modes, and the observed differences may include the effects of the labeling on the C-terminal Ala-344 carboxylate during the \(S\)-state cycling. However, we cannot analyze the details of the labeling effects on the spectral features at 1700–1500 cm\(^{-1}\).
cm\(^{-1}\) because the band intensity tends to fluctuate easily due to the presence of the large background from the water and amide absorptions.

S-state dependence of the carboxylate symmetric stretching bands of the D1 C-terminal Ala-344 was revealed more clearly in the L-[1-\(^{13}\)C]alanine/L-[1-\(^{12}\)C]alanine double difference spectra shown in Fig. 3, which were obtained by subtracting L-[1-\(^{13}\)C]alanine-labeled S-state difference spectra from the unlabeled S-state difference spectra, are shown.

FIG. 3. L-[1-\(^{12}\)C]alanine/L-[1-\(^{13}\)C]alanine double difference FTIR spectra for S-state cycling in the carboxylate symmetric stretching region (1500–1200 cm\(^{-1}\)). Double difference spectra for S\(_2\)/S\(_1\) (a), S\(_3\)/S\(_2\) (b), S\(_3\)/S\(_0\) (c), and S\(_0\)/S\(_3\) (d) differences, which were obtained by subtracting L-[1-\(^{13}\)C]alanine-labeled S-state difference spectra from unlabeled S-state difference spectra, are shown.

Effects of L-[1-\(^{13}\)C]Alanine Labeling on Low Frequency S\(_2\)/S\(_1\) FTIR Spectra—Fig. 4 shows the low frequency (670–350 cm\(^{-1}\)) S\(_2\)/S\(_1\) FTIR difference spectra of PS II core particles from the control (black line) and L-[1-\(^{13}\)C]alanine-labeled (red line) cells. The control spectrum agreed well with that reported previously (17) and showed bands at 652(\textpm{}), 642(+), 629(+), 617(\textpm{}), 606(+), 590(+), and 577(\textpm{}) cm\(^{-1}\) as well as many medium and low intensity bands. The bands at ~400(\textpm{}) cm\(^{-1}\) and 590(\textpm{}) cm\(^{-1}\) were ascribed to the ferricyanide added to the sample suspension as an electron acceptor and ferrocyanide formed by photoreduction of ferricyanide, respectively (26). Although the spectrum of L-[1-\(^{13}\)C]alanine-labeled particles was basically similar to the unlabeled control spectrum, small but distinctive differences between the two spectra were observed reproducibly at >590 cm\(^{-1}\). The intensity of the 617(\textpm{}) cm\(^{-1}\) band changed considerably, and the bands at 642(+), 629(+), and 617(\textpm{}) cm\(^{-1}\) were downshifted slightly upon the L-[1-\(^{13}\)C]alanine labeling. Most of the bands for S\(_2\)/S\(_1\) in the low frequency difference spectrum were significantly affected upon universal \(^{13}\)C labeling with the exception of the 577(\textpm{}) cm\(^{-1}\) band for the putative skeletal vibration of the manganese cluster (26). Therefore, it is conceivable that the \(\alpha\)-carboxylate in the C-terminal Ala-344 is responsible for low frequency bands affected by L-[1-\(^{13}\)C]alanine labeling. The low frequency result is compatible with the view that the \(\alpha\)-carboxylate of the D1 C-terminal Ala-344 associates with metal ion(s) as a direct ligand.
The present results clearly demonstrated that vibrations of the α-carboxylate in the D1 C-terminal Ala-344 changed characteristically during S-state cycling. As shown in Fig. 3, the bands from the α-carboxylate of Ala-344 appeared strongly in the double difference spectrum for S0/S1, with much reduced intensity in the double difference spectrum for S1/S2, and then appeared prominently again at slightly higher frequencies in the double difference spectrum for S2/S3 and S3/S0. Extended x-ray absorption fine structure measurements indicate that the core structure of the manganese/Ca2+ cluster remains almost same during the S1-to-S2 transition but changes considerably during the S2-to-S3 transition (28). Therefore, it is likely that the S-state-dependent changes in the vibrations of the α-carboxylate of the D1 C-terminal Ala-344 are not caused by S-state-dependent structural alterations in the manganese/Ca2+ cluster but are mainly due to changes in the oxidation state of the manganese ion(s) during S-state cycling. The present results indicate that the α-carboxylate of the D1 C-terminal Ala-344 structurally associates with a manganese ion that is oxidized during the S1-to-S2 transition and reversely reduced during the S2-to-S3 transition. The oxidation state of the manganese ions is thought to be MnIII/MnIII/MnIV/MnIV (or MnII/MnII/MnIII/MnIII) and MnIII/MnIV/MnIV (or MnIII/MnIII/MnII/MnIV) for the S1 and S2 states, respectively (29). Therefore, the α-carboxylate of Ala-344 is thought to structurally associate with a manganese ion oxidized from MnIII to MnIV during the S1-to-S2 transition. The spectral changes observed during S-state cycling strongly indicate that this oxidized manganese ion undergoes no redox change during the S2-to-S3 transition and then is reduced again from MnIV to MnIII during the S3-to-S0 transition and shows no redox change during the S0-to-S1 transition. A manganese ion probably becomes oxidized during the S2-to-S3 and possibly during the S3-to-S0 transitions (29). The appearance of few isotopic bands after the second and fourth flashes indicates that manganese ion(s), other than the ion associated with the α-carboxylate of Ala-344, are oxidized during the S2-to-S3 and S3-to-S0, and little change in structure of the α-carboxylate of Ala-344 occurs during these steps.

These interpretations are rationalized most straightforwardly by assuming direct ligation of the α-carboxylate of Ala-344 to the manganese ion. As pointed out by Chu et al. (16), the frequencies of the symmetric stretching bands of the α-carboxylate in the S0/S1 difference spectrum are compatible with those from a carboxylate that ligates a metal ion in an unidentate manner but are considerably different from a free and hydrogen-bonded carboxylate or a carboxylate ligating metal ion(s) in a bridging or a chelating manner. A carboxylate interacting with a non-metal atom through unusually strong hydrogen bond(s) could show bands at frequencies found in this study. However, this may be a less likely case because the spectral region for the Ala-344 α-carboxylate is not influenced by deuteration (23). Therefore, it is likely that the Ala-344 α-carboxylate ligates a manganese ion in an unidentate manner throughout S-state cycling, and this manganese ion is oxidized from MnIII to MnIV and reduced from MnIV to MnIII during the S1-to-S2 and S2-to-S3 transitions, respectively, as illustrated in Fig. 5. Taking into account the isotopic downshift of the symmetric carboxylate stretching modes by the 13C labeling of the Ala-344 α-carboxylate and presumed downshift induced by the oxidation of the manganese ion during the S1-to-S2 transition, the bands in the [1313C]/[1313C]alanine spectrum for the S2/S1 difference (Fig. 3a) at 1356(−), 1340(−), 1319(+), and 1304(−) cm⁻¹ can be assigned to S1 (13C), S2 (13C), and S3 (13C) modes, respectively. Accordingly, the bands for the S0/S3 difference (Fig. 3c) at 1360(+), 1344(−), 1323(−), and 1311(+) cm⁻¹ can be assigned to S0 (13C), S0 (13C), S3 (13C), and S3 (13C) modes, respectively. It is of note in this context that the isotopic bands in the double difference spectrum for the S2-to-S3 transition shifted upwards by ~4 cm⁻¹ as compared with those for the S1-to-S2 transition (Fig. 3). This suggests that the interaction between the C-terminal α-carboxylate and the redox-active manganese ion may be slightly weaker in the S1 and/or S2 state than that in the S1 and/or S3 state. The S3-to-S0 transition involves more drastic changes in the oxidation state and structure of the manganese/Ca2+ cluster (28, 29), and these may indirectly influence the C-terminal carboxylate mode. The direct ligation of the Ala-344 α-carboxylate to the manganese ion is also consistent with changes observed in the low frequency region (>590 cm⁻¹) of the S0/S1 difference spectrum after 13C labeling of the Ala-344 α-carboxylate shown in Fig. 4 because the bands for out-of-plane [π(COO)] or in-plane [δ(COO)] bending modes of a carboxylate that ligates a metal ion could appear in this frequency region (27, 30, 31). Such vibrational modes may be partly responsible for the marked 13C/13C isotopic bands induced by the universal 13C labeling of the OEC (26).

However, this interpretation conflicts with the 3.5 Å x-ray model of the S1 state OEC, in which the C-terminal carboxylate of Ala-344 is located close to the Ca2+ ion of the Mn4Ca4 cubane-like cluster core (13). We note in this context that the α-carboxylate is not resolved in any reported electron density map, presumably due to some radiation damage of crystals during data collection. Nevertheless, the present results may be alternatively interpreted by taking the 3.5 Å x-ray model into account as presented in Fig. 5 (in the parentheses). The C-terminal carboxylate of Ala-344 ligates the Ca2+ ion in an unidentate manner in the S1 state and unidentately ligates the MnIV ion, which was oxidized upon the S1-to-S2 transition, instead of the Ca2+ ion in the S3 state. The increase in the formal charge of the manganese ion from +3 to +4 upon the S2 state formation may preferentially facilitate ligation of the C-terminal carboxylate to the higher valence manganese ion. We may presume that change in a formal charge from +2 (Ca2+) to +4 (MnIV) by this metal exchange affects the stretching modes of the Ala-344 α-carboxylate, but details of the consequences of this model cannot be evaluated for the lack of available data obtained using compatible model complexes. The C-terminal carboxylate remains bound to the same MnIV ion during the S2-to-S3 transition, but the metal exchange occurs again during the S3-to-S0 transition, which results in binding of a Ca2+ ion instead of a manganese ion in the S0 state. This scheme is compatible with the x-ray model but is not easy to reconcile with the reports that the depletion of the functional Ca2+ ion from OEC does not induce drastic changes in mid-frequency S2/S1 difference spectrum (32, 33).

In the present study, we succeeded in following the changes in structural and/or chemical properties of a specific amino acid ligand, α-carboxylate of the D1 C-terminal Ala-344, for the manganese/Ca2+ cluster in OEC during S-state cycling for the first time. Apparently, higher quality x-ray crystallographic data are required for the elucidation of the present vibrational spectroscopic results on a strictly structural base.

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