Impact of Replacement of D1 C-terminal Alanine with Glycine on Structure and Function of Photosynthetic Oxygen-evolving Complex

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The C-terminal alanine 344 (Ala-344) in the D1 protein of photosystem II is conserved in all of the organisms performing oxygenic photosynthesis. A free α-COO⁻ of Ala-344 has been proposed to be responsible for ligating the Mn cluster. Here, we constructed a mutant having D1 in which D1-Ala-344 was replaced with glycine (Gly) in cyanobacterium Synechocystis sp. PCC 6803. The effects of this minimal change in the side group from methyl to hydrogen on the properties of the oxygen-evolving complex were comprehensively investigated using purified core particles. The mutant grew photoautotrophically, and little change was observed in the protein composition of the oxygen-evolving core particles. The Gly-substituted oxygen-evolving complex showed small but normal S2 multiline and enhanced g = 4.1 electron spin resonance signals and S2-state thermoluminescence bands with slightly elevated peak temperature. The Gly substitution resulted in distinct but relatively small changes in a few bands arising from the putative carboxylate ligand for the Mn cluster in the mid-frequency (1800–1000 cm⁻¹) S2/S1, Fourier transform infrared difference spectrum. In contrast, the low frequency (670–350 cm⁻¹) S2/S1, Fourier transform infrared difference spectrum was markedly changed by the substitution. The results indicate that the internal structure of the Mn cluster and/or the interaction between the Mn cluster and its ligand are considerably altered by a simple change in the side group, from methyl to hydrogen, at the C-terminal of the D1 protein.

Photosynthetic water oxidation takes place in an oxygen-evolving complex (OEC)¹ in which the catalytic center is composed of a tetranuclear Mn cluster located on the luminal side of the D1/D2 heterodimer. Two water molecules are oxidized to an oxygen molecule through five intermediates labeled Sn (n = 0–4), where n denotes the number of oxidizing equivalents stored. In a dark-adapted sample, a thermally stable S1 state predominates. The Sn state advances to the Sn+1 state by absorbing each photon to reach the highest oxidation state, S4, which spontaneously relaxes to the lowest oxidation state, S0, concomitant with the release of an oxygen molecule (1, 2).

Studies using chemical modifiers (3) and electron spin echo envelope modulation (4) and FTIR spectroscopy (5, 6) suggested that histidine and/or acidic amino acids are involved in the ligation of the Mn cluster. Several residues of the D1 protein have been proposed to have been based on site-directed mutagenesis studies mainly using cyanobacterium Synechocystis sp. PCC 6803 as potential candidates for the ligands to the Mn cluster, (reviewed in Refs. 7–9). They are Asp-170, Glu-189, His-190, His-332, Glu-333, His-337, Asp-342, and Ala-344 (10–15), some of which were arranged in close proximity to the Mn cluster in x-ray structural models of photosystem (PS) II (16–19). However, the properties of OEC have not been characterized using isolated PS II preparations with the exception of a few mutants. The studies using the O2-evolving PS II core particles from the D1-D170H mutant showed that the mutation leads to little change of the S2 and S2 multiline ESR, S2 multiline electron spin echo modulation signals, and the mid-frequency S2/S1 FTIR difference spectrum but does lead to some changes of the low frequency (650–500 cm⁻¹) S2/S1 FTIR difference spectrum (20, 21). The PS II cores from D1-H332E showed no O2 evolution but retained the Mn cluster with an altered S2 multiline ESR signal in which the electron spin echo envelope modulation spectrum showed no nitrogen modulation (22, 23). The PS II cores from D1-E189D, D1-E189N, D1-E189H, D1-E189G, and D1-E189S showed no oxygen evolution and neither S1 nor S2 multiline ESR signal but did reveal a Y2S2-state split signal. In contrast, D1-E189Q and D1-E189L mutants grew photoautotrophically, and their PS II cores showed the normal multiline signals (24).

The D1 protein is synthesized with a short C-terminal extension with the exception of Euglena, assembled into the PS II complex (25), and subsequently cleaved on the carboxyl side of Ala-344 by D1 C-terminal-processing protease (26). The processing is prerequisite to the light-dependent assembly of the Mn cluster (27, 28), but the mutant with no extension by substituting the stop codon at D1–345 for the amino acid codon (D1–345stop) showed normal photoautotrophic growth and O2 evolution capability (11, 29). Replacement of D1-Ala-344 with Gly, Met, Ser, or Val in the D1–345stop (D1-Ala-344-stop) strain did not affect photoautotrophic growth. However, Tyr or

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Lys substitution led to a marked decrease in O₂ evolution and loss of the capability for photoautotrophic growth (11). None of D1 C-terminal-truncated mutants evolved oxygen (11). Therefore, the free C-terminal(α-COO⁻) of the D1 Ala-344 has been proposed to ligate one or more Mn ions (11). The PS II core particles isolated from wild-type Synechocystis cells labeled with 1-13C alanine showed that several bands in the mid-frequency S₂/S₁ FTIR difference spectrum are affected by the labeling (59). This result indicates that the isotope-affected bands can be ascribed to the α-carboxylate group of D1-Ala-344 as a ligand for the Mn cluster, although an indirect structural coupling between the Mn cluster and D1-Ala-344 cannot be excluded. Ligation of the D1-Ala-344 carboxylate to the Mn cluster was proposed based on the 3.6 Å (17) and 3.7 Å (18) x-ray structural model. The C-terminal carboxylate was arranged in close proximity to a Ca ion in a recent 3.5 Å model in which the cubane-like M₆CaO₄ linked to a fourth Mn by a mono-μ-oxo bridge was proposed, although the C-terminal carboxylate was disordered and not visible in the electron density map (19). It may be worthwhile to note in this context that no individual metal ion in the OEC was resolved in any reported x-ray electron density maps.

The D1-A344G mutant has been reported to grow photoautotrophically (11). Although the replacement of alanine with glycine is accompanied with only the change of a side group from methyl to hydrogen, the functional and/or structural properties of the Mn cluster may be affected even by this minimal change if the C-terminal free carboxylate is involved in the ligation of the Mn cluster. In the present study, we have constructed D1-Ala-344-stop (= D1-Ser-345stop) and D1-A344G-stop mutants on a Synechocystis sp. PCC 6803 strain with histidine-tagged CP47 and have isolated active PS II core particles from them. The replacement of the C-terminal Ala with Gly had little effect on biochemical properties of OEC but led to considerable changes in functional/structural properties of the Mn cluster. These results are compatible with the proposal that the C-terminal α-COO⁻ carboxylate of D1-Ala-344 is crucial for maintaining the infrastructure of the Mn cluster, possibly by ligating the Mn cluster.

**EXPERIMENTAL PROCEDURES**

**Construction of a Host Strain for Mutation**—The genomic DNA from *Synechocystis* sp. PCC 6803 was amplified by PCR with a specific primer set corresponding to the *psbA2*, *psbA2*, or *psbA3* gene. For *psbA1*, a 1727-bp DNA containing 1080 bp *psbA1* plus 414 bp 5' and 233 bp 3' flanking DNA was cloned into the plasmid PBLeucist II SK⁺. A 0.45-kb HindII/NsiII fragment containing 40% *psbA1* was replaced by a 1.4-kb fragment from the plasmid pACYC184 conferring resistance to chloramphenicol to generate the plasmid pNA1. For *psbA2*, a 1972-bp DNA containing 1053 bp 3' 97% *psbA2* plus 919 bp 3' flanking DNA was cloned into pUC19 to generate the plasmid pNA2. A 0.9-kb HindII/StuI fragment corresponding to 83% *psbA2* and a part of its downstream gene, *slr1312*, was replaced by a 2-kb fragment of the plasmid pRLA63 (a kind gift from Prof. T. Omata, Nagoya University) conferring resistance to spectinomycin to generate the plasmid pNA2. For *psbA3*, a 1691-bp DNA containing 1080 bp *psbA3* plus 326 bp 5' and 285 bp 3' flanking DNA was cloned into the plasmid pUC19. A 0.4-kb KpnI/KpnI fragment was replaced by a 1.7-kb fragment of pACYC184 conferring resistance to tetracycline to generate the plasmid pNA3. The plasmids pNA1, pNA2, and pNA3 were successively transformed into the glucose-tolerant wild-type strain of *Synechocystis*. The resulting strain (N3AA) was resistant to chloramphenicol, tetracycline, and spectinomycin and lacked all three *psbA* genes. A hexahistidine tag was introduced to the C terminus of CP47 of the wild-type and N3AA strain as described previously (31) to generate the wild*-type and B-His/N3AA strain. The B-His/N3AA strain was used for further site-directed mutagenesis.

**Construction of Site-directed Mutants**—A 1.3-kb fragment from the plasmid pUC4K conferring resistance to kanamycin (Km) was inserted into the *StuI* site located 288 bp downstream of the *psbA2* gene in pNA2, thus generating the plasmid pNA219. Mutations were intro-

duced into the *psbA2* gene in pNA219 using a commercial oligonucleotide-mediated mutagenesis kit (QuickChange site-directed mutagenesis kit; Stratagene). For the Ala-344-stop (= Ser-345stop) strain, the Ser-345 codon TCT was changed to a stop codon TGA. For the A344G-stop strain, the Ala-344 codon GCC and the Ser-345 codon TCT were changed to glycine GGC and the stop codon TGA, respectively. Plasmids bearing the mutations were transformed into the host strain (B-His/ N3AA), and single colonies were selected for their photoautotrophic growth ability on solid BG-11 medium containing 5 μg/ml kanamycin.

**Culture Conditions and Preparation of PSII Core Particles**—The *Synechocystis* cells were photoheterotrophically grown in liquid BG-11 medium supplemented with 5 mM glucose at 30 °C under 30–50 μmol photon/m²s in an 8-liter Clearboy (NALGENE), bubbling with air up to 0.5 volumes of Chl/ml unless otherwise noted. The PS II core particles were prepared as previously described (31). Briefly, the harvested cells were disrupted using a Bead beater (Bio-Spec Products). The resulting thylakoid membranes were solubilized using n-dodecyl-β-D-maltoside, and then the PS II particles were affinity purified with a nickel-nitri-

triacetic acid column (Qiagen). The purified core particles were washed with a medium (medium A) containing 400 mM sucrose, 20 mM NaCl, 20 mM CaCl₂, and 20 mM Mes-NaOH (pH 6.0) supplemented with 10% (v/v) polyethylene glycol 6000 and suspended in medium A after extensive washing with medium A.

**Protein Composition**—The PS II core particles were solubilized using 1% SDS and then electrophoresed in an SDS-PAGE with a 16–22% gradient gel containing 7.5 × urea (32). A sample corresponding to 0.8 μg of Chl was applied to each lane. Peptide bands were visualized by staining with Coomassie Brilliant Blue R-250. The apparent molecular mass of a resolved protein was estimated by comigrating a molecular mass standard (Bio-Rad).

**Measurements**—Mid-frequency (1800–1000 cm⁻¹) FTIR spectra were recorded on a BrukerIFS 66/s FTIR spectrophotometer equipped with a mercury cadmium telluride detector (EG&G Optoelectronics D316/6). Low frequency (650–350 cm⁻¹) FTIR spectra were recorded on a Bomen MB102 spectrophotometer equipped with a Si bolometer (Infrared, HDL-5) as previously described (31). The PS II core suspension (20 μg of Chl) was mixed with 1 μl of sodium ferricyanide solution (100 mM sodium 5-cyano-5 ′-isothiocyanato-1-methyl-4-hydroxy-2-pyridone) and 200 μM ferricyanide. The sample suspension was diluted to either a 20-mm BaF₂ disk (mid-frequency) or an AgCl disk (low frequency) were partially desiccated and then rehydrated. The sample temperature was maintained within ± 0.03 °C using a homemade cryo-

ostat. The sample cores were illuminated with a flash provided from a frequency-doubled Nd³⁺:YAG laser (Spectra Physics INDI-50, 532 nm, pulse width 6–7 ns) with flash energy of 10 mJ/cm² at the sample surface. Single beam spectra were accumulated at 4-cm⁻¹ resolution for 15 s in the mid-frequency (20 scans) or in the low frequency region (10 scans) before and after excitation, and light – dark spectra were cal-

culated. 116–145 mid-frequency difference spectra or 271–284 low fre-

quency difference spectra were averaged.

**Low temperature X-band ESR spectra**—were measured using a Bruker ESP 5800 spectrometer equipped with an Oxford-900 cryostat and a temperature controller Oxford, (Oxford). The sample cores (4 mg of Chl/ml) in a Spracil quartz ESR sample tube were illuminated at 213 K for 3 s with a cold light (Hayashi, LA-150TX) passing through a long pass filter (≥680 nm). Thermoluminescence was measured using a home-

made apparatus (33). The cells were suspended in medium A at 250 μg of Chl/ml in the presence or absence of 0.1 mM DCMU. The sample suspension was illuminated at 0 °C with a saturating xenon flash. The O₂ evolution activity was measured using a Clark-type oxygen electrode in medium A at 25 °C under saturating light conditions supplemented with 0.03% of glycerol. The oxygen evolution rates were averaged.

**RESULTS**

**Physiological and Biochemical Properties**—As shown in Table I, the histidine-tagged wild-type (wild*-type) and the C-terminal extension-truncated Ala-344-stop cells grew photoautotrophically under both low and high light conditions and evolved oxygen at rates similar to the wild-type cells. The affinity-purified PS II core particles from wild*-type and Ala-

344-stop cells showed high O₂ evolution capability, although the activity of the Ala-344-stop cores was slightly lower than that of the wild*-type cores, following the trend observed for O₂ evolution in the cells. The A344G-stop cells grew photoautotrophically under low light conditions as previously reported.
(11) and evolved oxygen at \(-90\%\) of the rate of the Ala-344-stop cells but did not grow photoautotrophically under high light conditions. The PS II core particles from the low light-grown A344G-stop cells preserved high \(O_2\) evolution activity, but it was relatively lower \((-60\%)\) than that from the Ala-344-stop cells. The activity was not enhanced by the further supplementation of the \(Ca^{2+}\) and/or \(Cl^-\) to medium A.

Fig. 1 shows SDS-PAGE profiles of the PS II core particles from wild\(^*\)-type \((a)\), Ala-344-stop \((b)\), and A344G-stop \((c)\) cells of Synechocystis sp. PCC 6803. An 0.6-\(\mu\)g Chl-containing sample was applied to each lane. The positions of molecular mass standards and PSII proteins are indicated in the left and right margins, respectively. See “Results” for a protein band marked by asterisk. Wild\(^*\)-type represents a wild-type strain with a histidine tag.

### Structural Properties of the Mn Cluster

Fig. 3 shows the light-induced ESR spectra of the PS II core particles from Ala-344-stop \((a)\) and A344G-stop \((b)\) cells of Synechocystis sp. PCC 6803. Instrument settings: temperature, 6 K, microwave power, 0.5 mW; microwave frequency, 9.5 GHz; modulation frequency and amplitude, 100 kHz and 1.6 millitesla, respectively.

### Table I

| Strains          | Photoautotrophic growth | Oxygen evolution* |
|------------------|-------------------------|-------------------|
|                  | Low light* | High light* | Cells | PSII cores |
| Wild-type        | + + + +   | ++ + +     | 410 (100)* | n.d. |
| Wild\(^*\)-type | + + + +   | ++ + +     | 220 (100) | 2400 (96) |
| Ala-344-stop     | + + + +   | ++ + +     | 395 (96)  | 2400 (96) |
| A344G-stop       | ++ +     | --         | 305 (85)  | 1450 (58) |

* \(\mu\)mol of \(O_2\) (mg of Chl)\(^{-1}\) h\(^{-1}\).
* 50 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\).
* 200 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\).
* Numbers in parentheses represent the relative \(O_2\)-evolving activity in relative %.

*Fig. 2. Thermoluminescence glow curves of wild-type \((a)\), wild\(^*\)-type \((b)\), Ala-344-stop \((c)\), and A344G-stop \((d)\) cells of Synechocystis sp. PCC 6803 in the presence \((A)\) or absence \((B)\) of 0.1 mm DCMU for generating \(S_2Q_A^-\) or \(S_2Q_B^-\) pair. Wild\(^*\)-type represents a wild-type strain with a histidine tag.*

*Fig. 3. Light – dark ESR spectra of the PS II core particles from Ala-344-stop \((a)\) and A344G-stop \((b)\) cells of Synechocystis sp. PCC 6803. The sample cores were illuminated at 213 K, a temperature at which the accumulation of the \(S_2\) state is allowed. The Ala-344-stop particles showed a prominent \(g = 2\) \(S_2\) multiline, a much smaller \(g = 4.1\) \(S_2\) signal, and a \(Fe^{2+}Q_A^-\) signal at \(g = 1.9\). The spectral features of the observed \(S_2\) ESR signals were very similar to those in the normal OEC. The A344G-stop spectrum showed a smaller multiline and much larger \(g = 4.1\) signals compared with the Ala-344-stop spectrum, although the hyperfine structure of the multiline signal and the width and position of the \(g = 4.1\) signal were almost identical to those of the Ala-344-stop spectrum. These results indicate that the Gly substitution in-
spectrum. Several of the bands in the symmetric carboxylate
as can be clearly observed in the double difference spectrum (blue line)
but distinctive difference from the Ala-344-stop one (red line). The double
difference spectrum (c) was obtained by subtracting the A344G-stop
spectrum from the Ala-344-stop spectrum after normalization with
respect to the peak-to-peak intensity of the 2115(−)-cm⁻¹ terricyanide
and 2034(+)-cm⁻¹ ferrocyanide bands (31). A dark − dark spectrum (d)
is presented to show the noise level.

Fig. 4 shows the mid-frequency (1800–1000 cm⁻¹) S₆/S₁ FTIR difference
spectra of the PS II core particles from wild-type (a), Ala-344-stop (b, blue
line), and A344G-stop (b, red line) cells of Synechocystis sp. PCC 6803.
Double difference spectrum (c) was obtained by subtracting the A344G-
stop spectrum from the Ala-344-stop spectrum after normalization with
respect to the peak-to-peak intensity of the 2115(−)-cm⁻¹ terricyanide
and 2034(+)-cm⁻¹ ferrocyanide bands (31). A dark − dark spectrum (d)
is presented to show the noise level.

Fig. 5 shows the low frequency (670–350 cm⁻¹) S₆/S₁ FTIR difference
spectra of the PS II core particles from the Ala-344-stop (a, blue line) and
A344G-stop (a, red line) cells and the double difference spectrum (b). The A344-stop S₆/S₁ spectrum showed prominent bands at 629(+), 617(−), 606(+),
590(+), 577(−), 403(−), and 388(−)-cm⁻¹ as well as many other
medium to low intensity bands. The 590(+) and 400(−)-cm⁻¹ bands were ascribed to the vibrational modes of ferrocyanide and ferricyanide, respectively (31). The spectrum was most comparable with the previously reported wild-type spectrum measured under different sample conditions, but bands at 660(+), 652(−), 642(+), 374(+), 368(−), and 359(−)-cm⁻¹ had not been well resolved because of the large absorption of water (31). The spectrum of A344G-stop (a, red line) was markedly different from that of Ala-344-stop. The double difference spectrum (b) showed prominent bands at 663(+), 648(+), 625(−), 615(−), 606(+), 384(+), 368(−), and 361(+)-cm⁻¹ as well as several minor bands at 532 cm⁻¹ and in the 500–420 cm⁻¹ region. Based on a study using ¹⁸O water, it has been suggested that the Mn-O-Mn cluster modes in the S₂ and S₁ states are
responsible for the 606(+) and 625(−)-cm⁻¹ bands (34). Therefore, the results indicate that the interactions between Mn ions in the cluster are considerably influenced by the Gly substitution. However, the Gly substitution was not observed to affect the bands at 577(−), 565(+), 554(+), and 542(+) cm⁻¹. The 577(−)-cm⁻¹ band has been ascribed to the skeletal vibration of the Mn cluster or the Mn-ligand(oxygen) interaction (31). Therefore, the primary structure of the Mn cluster is thought to be little affected by the Gly substitution.
Impact of Gly Substitution at D1 C-terminal on OEC

The present results showed that the A344G-stop mutant grew photoautotrophically with a high O₂ evolution activity and revealed no biochemical difference in the isolated PS II core complex. However, the mutant cells could not grow photoautotrophically under high light conditions. The elevated peak temperature of the S₂-state thermoluminescence bands in the A344G-stop cells indicates that the oxidation potential of the S₂-state Mn cluster is lower in the mutant than in the control cells. This may account for the increased susceptibility of the A344G-stop cells to high light because this change lowers the efficiency for the oxidation of the substrate water and, consequently, leads to the increase in the charge recombination probability in PS II, promoting the generation of the reactive oxygen species.

The Ala-344-stop – A344G-stop double difference spectrum (Fig. 4c) showed the bands at 1403–1345 and 1565–1503 cm⁻¹ in the carboxylate symmetric and asymmetric stretching regions, respectively. The band change induced by the Gly substitution is explained by assuming a shift of a single carboxylate group. As shown in Fig. 6, the observed double difference spectrum was reproduced by assuming the downshift of an S₂/S₁ differential pair for Ala-344-stop (a and b, red lines) and A344G-stop (a and b, red lines), respectively. Two likely schemes of four possible schemes are presented. See “Discussion” for further details.

DISCUSSION

Fig. 6. Simulation of the Ala-344-stop/A344G-stop double difference FTIR bands. Experimentally obtained (c, dotted line) and simulated (c, solid line) double difference spectra. An S₂/S₁ differential band pair for Ala-344-stop (a and b, blue lines) and A344G-stop (a and b, red lines), respectively. Two likely schemes of four possible schemes are presented. See “Discussion” for further details.

Most of the bands in the low frequency S₂/S₁ difference spectrum were changed upon Gly substitution as seen in Fig. 5. The affected bands possibly included the S₂/S₁ modes of the Mn-O-Mn stretching vibration of the Mn cluster at 606(±2/ 625(−)) cm⁻¹ (34), Mn-COO⁻ bending modes of putative carboxylate ligands (36), the ring torsion mode of histidine residues (37), the amide IV (40% O = C-N bending) and amide VI (C = O bending) modes of polypeptide backbone (38), and ligand-dependent Mn-O modes at 532–420 cm⁻¹ (31, 39). The Gly substitution resulted in marked changes in the bands in the 400–500 cm⁻¹ region, frequencies that include the stretching vibrations between the Mn ion and the ligands (O and/or N) (40). These marked spectral changes upon Gly substitution suggest a gross structural change in the Mn cluster. Nevertheless, there are relatively insignificant changes in the mid-frequency region of the spectrum except for the putative Asp-342 mode. Interestingly, the D1-D170H OEC Synechocystis showed prominent changes in the low frequency S₂/S₁ spectrum (660–500 cm⁻¹) but limited changes in the mid-frequency (1800–1200 cm⁻¹) spectrum (20). A possible explanation for the different manifestation of the Gly substitution in the mid- and low frequency spectra is that the mid-frequency bands include the modes, which are not the direct ligands to the Mn cluster. Such indirect structural coupling may be less sensitive to intrastructural changes of the Mn cluster. The observed structural changes of the Mn cluster induced by the Gly substitution suggest that Ala-344 is crucial for maintaining the intrastructure of the Mn cluster as a ligand. Nevertheless, the present FTIR results do not preclude the possibility that the C-terminal Ala-344 carboxylate is located close to the Ca ion of the Mn₃CaO₄ cubane-like cluster core as proposed by the recent x-ray structural model (19). In this case, the present results suggest that Ca may participate in controlling the structural changes of the Mn cluster during the S₁ to S₂ transition and the C-terminal Ala is involved in this Ca-dependent function, although it is not clear how it is achieved at present.
As shown in Fig. 3, ≤70% of the total A344G-stop OEC existed in the S = 5/2 g = 4.1 state, whereas more than 90% OEC existed in the S = 1/2 multiline state in the control Ala-344-stop. Some difference in the electronic structure within the Mn cluster has been proposed to be responsible for the appearance of these two states (41, 42). The change in the intrastructure of the Mn cluster may induce considerable alterations in the low frequency modes of the Mn cluster with much smaller or little change in the mid-frequency modes indirectly coupled with the Mn cluster. The Sr²⁺-substituted OEC with an enhanced g = 4.1 signal showed the normal-like mid-frequency S₂/S₁ difference spectrum (43) and the markedly altered low frequency spectrum (34). Furthermore, little change of the mid-frequency S₂/S₁ spectrum has been reported upon conversion from the multiline to the 4.1 state by IR illumination (44). A possible difference between these two S₂ ESR states is a valence exchange between strongly antiferromagnetic Mn(IV) and Mn(III) (41, 42). The valence exchange may alter the Mn-Mn and/or Mn-ligand interactions that affect the low frequency modes but scarcely influence the mid-frequency modes.

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