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^{13}C ENDOR Reveals That the D1 Polypeptide C-Terminus Is Directly Bound to Mn in the Photosystem II Oxygen Evolving Complex

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Extensive research efforts have focused on characterizing the structure and ligand environment of the [Mn_4Ca] cluster that makes up the catalytic core of the oxygen-evolving complex (OEC) in photosystem II (PSII). The OEC catalyzes the light-induced four-electron oxidation of two molecules of H_2O to O_2 and 4 H^+ via a five-step reaction pathway referred to as the “S state” or “Kok” cycle.[1] When the OEC is poised in the S_2 state, EPR reveals a $g = 2$ “multiline” signal with ≈19 ^{55}Mn hyperfine lines resolved in the X-band CW spectrum.[2] This signal is attributed to an $S = {}^1/_2$ ground state arising from antiferromagnetic coupling of the four manganese ions and is reminiscent of EPR spectra of oxido bridged $Mn^{III}Mn^{IV}$ dimers. One such spectroscopic model of the OEC was the subject of a pulse EPR study in which electron nuclear double resonance (ENDOR) and electron spin−echo envelope modulation (ESEEM) spectroscopies probed water and methanol ligation to the Mn dimer core.[3] Those results provided a basis for the exploration of water and methanol binding to biological Mn clusters.[3,4]

In the present study, ENDOR spectroscopy is used to characterize the interaction of carboxylate ligands with the Mn ions of exchange-coupled clusters. Figure 1A presents the Q-band (34 GHz) Mims ENDOR spectrum of ^{13}C ($I = {}^1/_2$) in the carboxylate position of an acetate bridge between a Mn^{III} and Mn^{IV} in [$Mn^{III}Mn^{IV}(\mu\text{-}O)_2\mu\text{-}OAc(TACN)_2$]$(BPh_4)_2$ (**1**) (TACN = 1,4,7-triazacyclononane). This acetate moiety is invoked as a model for protein-derived carboxylates thought to coordinate the OEC in PSII.[5] We compare these ^{13}C-**1** results to ENDOR spectra of PSII from *Synechocystis* sp. PCC 6803 for which (i) the C-terminal α-COO^- group (Ala344) of D1 and all alanine-derived C═O groups are labeled with ^{13}C (α-^{13}C-Ala PSII; Figure 1B) and (ii) all C atoms are uniformly labeled with ^{13}C (Figure 1C).

The CW EPR spectra of the ^{13}C and ^{12}C acetate isotopologues of **1** are essentially identical (Figure S1). While ^{12}C-**1** gives no ENDOR intensity around the ^{13}C Larmor frequency ($\nu_L(^{13}C) = 13.0$ MHz at $B_0 = 1218.0$ mT), the ^{13}C-**1** spectrum possesses a pair of structured peaks centered at $\nu_L(^{13}C)$ and split by 1.9 MHz (Figure 1A). The dipolar ($\tilde{a}_{dip}$) and isotropic (a_{iso}) hyperfine interactions govern the magnitude of the splitting between the ENDOR peaks. $\tilde{a}_{dip}$ is defined as the site-specific through-space interaction of the ^{13}C nuclear spin with the electron spin on each Mn ion. a_{iso} originates from the unpaired electron spin population in the valence s-orbital of the ^{13}C atom. For multimetal clusters such as **1**, exchange interactions between the metal ions lead to an effective spin ($S = {}^1/_2$) that is different from the spins of the isolated ions ($S_{Mn^{III}} = 2$ and $S_{Mn^{IV}} = {}^3/_2$). Thus the observed hyperfine ($A_{iso}\tilde{1} + \tilde{A}_{dip}$) must be scaled by the appropriate projection factors to give a_{iso} and $\tilde{a}_{dip}$ before deriving both the covalent character of the bond and the distance between the unpaired spin for each site and the magnetic ligand nucleus. The projection factors for the Mn^{III} and Mn^{IV} ions in **1** are +2 and −1, respectively.[3] The site $\tilde{a}_{dip}$ representing each Mn•••^{13}C interaction was computed using the coordinates from the high-resolution X-ray crystallographic structure.[6] These two hyperfine contributions were then scaled by the projection factors and transformed into a common frame to give $\tilde{A}_{dip}$.[3] With the effective $\tilde{A}_{dip}$ contribution fixed, the value for A_{iso} was varied from +4 to −4 MHz until a satisfactory simulation of the experimental data was achieved. To resolve the structure evident in the ENDOR features of all spectra in Figure 1, the simulations required an isotropic line width parameter no larger than 0.2 MHz.[7] We also accounted for τ-dependent blind spots inherent to Mims ENDOR spectra.[8] Using this procedure, the simulation parameters that gave the best fit to the experimental data of ^{13}C-**1** were $\tilde{A}_{dip} = [-2.4, -0.8, 3.2]$ MHz and $A_{iso} = -1.0$ MHz (Figure 1A). This value for A_{iso} is small compared to that found for a carbon bound directly to a paramagnetic center,[9] as the α-C of the acetate is two bonds away from each Mn ion.

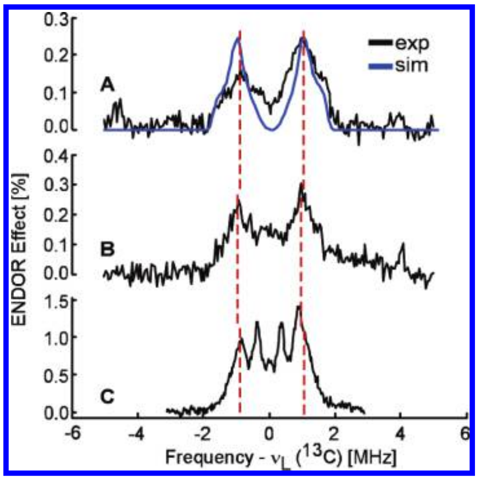

Figure 1. Q-band Mims ENDOR spectra (black) of (A) ^{13}C-**1**; (B) α-^{13}C-Ala PSII; (C) ^{13}C-PSII. Experimental parameters: $\nu_{MW} = 33.9$ GHz; $B_0 = 1218-1220$ mT; selected τ values from 180 to 300 ns were used (see Supporting Information); $t(\pi/2) = 20$ ns; $t(\pi_{RF}) = 64$ μs; $T = 12$ K (^{13}C-**1**) and 5 K (PSII). Simulation (blue) parameters for ^{13}C-**1** spectrum: $\tilde{A}_{dip} = [-2.5, -0.8, 3.2]$; $A_{iso} = -1.0$ MHz; line width 0.2 MHz.

Biochemical and spectroscopic studies[5] and X-ray crystallography[10–12] identify a number of residues as possible ligands to the Mn ions in the OEC. However, these studies give somewhat conflicting results. For example, D1-Ala344 is positioned differently in each X-ray structure. The Ferreira structure shows the COO^- group of the D1 polypeptide bound to the Ca ion, while structures

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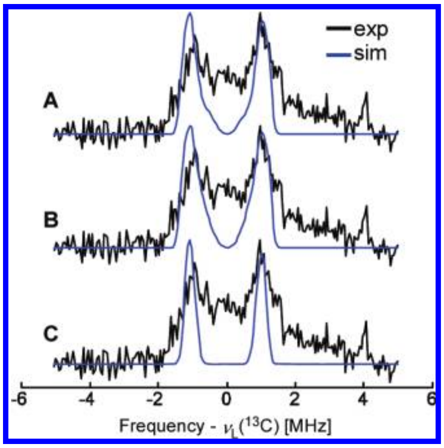

Figure 2. Q-band Mims ENDOR spectrum of α-^{13}C-Ala PSII (black) with simulations (blue) generated using coordinates from selected X-ray data of *T. elongatus* PSII. Simulation parameters: line width 0.2 MHz (A) Loll, $A_{\mathrm{iso}} =$ 1.2 MHz, $\tilde{A}_{\mathrm{dip}} = [-2.2, 0.8, 1.4]$; (B) Guskov, $A_{\mathrm{iso}} = 1.0$ MHz, $\tilde{A}_{\mathrm{dip}} = [-2.0, -1.5, 3.5]$; and (C) Ferreira, $A_{\mathrm{iso}} = 2.0$ MHz, $\tilde{A}_{\mathrm{dip}} = [-0.4, -0.2, 0.6]$.

provided by Loll and Guskov position it as a ligand to $\mathrm{Mn_B}$ (Figure S3). Fourier transform infrared (FTIR) difference results indicate that D1-Ala344 is not bound to Ca but is instead bound to a Mn ion.[13] Such discrepancies between crystallographic data in comparison to spectroscopy are perhaps not surprising as the X-rays used to collect the diffraction data cause reduction of all Mn ions to $\mathrm{Mn^{II}}$ and resultant changes to the coordination geometry.[14] Therefore, nondestructive spectroscopic methods like FTIR and EPR must also be utilized to characterize the native ligand environment of the $[\mathrm{Mn_4Ca}]$ cluster.

The ENDOR spectrum of α-^{13}C-Ala PSII is presented in Figure 1B. While all alanines are isotopically labeled, only D1-Ala344 is found close enough to the Mn cluster to have a non-negligible hyperfine coupling.[15] This spectrum exhibits a splitting pattern similar to that observed for ^{13}C-**1**. Nuclear coordinates needed to compute $\tilde{A}_{\mathrm{dip}}$ for the OEC$\cdots$D1-Ala344 interaction were taken from the *T. elongatus* PSII X-ray structures of Loll, Guskov, and Ferreira. The projection factors used were those reported in ref 16, assuming that $\mathrm{Mn^{III}}$ is at the $\mathrm{Mn_C}$ position (Figure S3). Our choice for the position of the $\mathrm{Mn^{III}}$ ion is based on multifrequency ESEEM results that suggest D1-His332 is bound to the $\mathrm{Mn^{III}}$ ion in the $\mathrm{S_2}$ state of the OEC.[17] However, spectra using alternate sets of projection factors were also simulated which are consistent with the conclusions (see Supporting Information). With $\tilde{A}_{\mathrm{dip}}$ fixed for each X-ray structure, the value of A_{iso} needed to simulate the α-^{13}C-Ala PSII data was determined to be 1.2, 1.0, and 2.0 MHz, respectively (Figure 2). The longer distance between D1-Ala344 and the Mn ions in the Ferreira structure corresponds to a small $\tilde{A}_{\mathrm{dip}}$ and thus a larger A_{iso} must be used to provide the proper peak splitting, and even then the peaks are quite narrow and featureless compared to the experimental spectrum. However, with D1-Ala344 directly bound to Mn in the Loll and Guskov structures, a larger $\tilde{A}_{\mathrm{dip}}$ is computed, requiring a smaller A_{iso}, leading to a better fit of the structural features in Figure 2. Based on our analysis of the ENDOR data of ^{13}C-**1**, which possesses an intimate Mn$\cdots$COO$^-$ interaction, we regard any value for an A_{iso} significantly larger than 1.2 MHz for a Mn-coordinating carboxylate moiety as unlikely. Furthermore, we expect an $A_{\mathrm{iso}} \approx 0$ MHz for a ligand bound only to the diamagnetic Ca ion, as there would be no unpaired electron spin density at the nucleus.[18] Considering this, we conclude that the C-terminal carboxylate of D1-Ala344 is a Mn ligand and cannot be bound to only the Ca ion.

The ENDOR spectrum of uniformly ^{13}C-labeled PSII (Figure 1C) also shows prominent peaks split by ≈1.7 MHz, a slightly smaller splitting than observed for the α-^{13}C-Ala PSII sample (Figure 1B). We note that if the intensity of the uniformly labeled sample is appreciably more intense than that for the α-^{13}C-Ala sample. This implies that there are multiple ^{13}C-containing moieties with couplings in the 1.7−1.9 MHz range. The slightly larger splitting in the α-^{13}C-Ala PSII spectrum indicates the OEC Mn interaction with the D1-Ala344 ^{13}C is one of the strongest (see red line Figure 1). An additional set of peaks with a resolved splitting of 0.9 MHz arises from another group of ^{13}C nuclei with appreciably weaker couplings. A number of mutagenesis, FTIR difference and X-ray crystallographic studies suggest additional amino acid derived carboxylate ligands to the OEC (see Figure S3).[5,10,11] Further ^{13}C ENDOR studies are currently underway to identify which of these amino acids are responsible for these more weakly coupled features.

In conclusion, ^{13}C ENDOR data indicate that multiple classes of ^{13}C-containing moieties are coupled to the $[\mathrm{Mn_4Ca}]$ cluster. In particular, these data conclusively show that the C-terminal carboxylate of D1-Ala344 is bound to at least one Mn ion.

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Supporting Information Available: Supporting spectra, simulations, and experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org

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