The Mn_4Ca Cluster in PSII

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ESEEM Spectroscopy Reveals Carbonate and an N-Donor Protein-Ligand Binding to Mn^{2+} in the Photoassembly Reaction of the Mn_4Ca Cluster in Photosystem II**

*Jyotishman Dasgupta, Alexei M. Tyryshkin, and G. Charles Dismukes**

The oxidation of water to dioxygen gas is an energy demanding and mechanistically complex chemical reaction. These limitations have restricted the development of practical water-splitting catalysts needed for renewable hydrogen production. By contrast in photosynthesis, water oxidation proceeds efficiently using visible light energy that is absorbed by chlorophyll photopigments and made chemically available through charge separation in the protein complex, photosystem II (PSII).[1] Water is oxidized and O_2 evolved ultimately at an inorganic active site ($Mn_4O_xCa_1Cl_y$)[2,3] known as the water oxidizing complex (WOC), by a mechanism that is debated.[4,5] X-ray structural evidence suggesting that inorganic carbon in the form of (bi)carbonate may serve as another required inorganic cofactor within the WOC has been circumstantial[2] and disputed.[6] However, studies have shown that (bi)carbonate unquestionably participates in the light-driven assembly of the inorganic core starting from the cofactor-depleted apo-WOC-PSII protein (called photoactivation). This is seen both by its acceleration of the rate of Mn^{2+} photooxidation,[7,8] and by EPR spectroscopy of the resulting Mn^{3+} assembly intermediate which revealed a strong influence on the strength of both the ligand field and the ^{55}Mn magnetic hyperfine coupling.[9] Although these observations indicate that (bi)carbonate acts to significantly alter the structural environment of the first Mn^{3+} formed during photoassembly of the cluster and its electrochemical potential, they have failed to identify where it actually binds within PSII. Thus, no direct evidence exists for the postulated inner coordination complex between bicarbonate and Mn^{2+} during photoactivation. Herein, we provide evidence from electron spin echo envelope modulation (ESEEM) spectroscopy[10–12] for a direct magnetic hyperfine coupling between ^{13}C-bicarbonate and the Mn^{2+} precursor to the photooxidized Mn^{3+} formed in the first step of the photoactivation process.

The photoactivation process occurs spontaneously upon illumination of the cofactor depleted apo-WOC-PSII protein in the presence of the free cofactors (Mn^{2+}, Ca^{2+}, Cl^-), visible light, and an electron acceptor.[13] Kinetic analysis of the assembly process has shown that it occurs in two resolved steps. The first intermediate is described by the binding of one Mn^{2+} ion and its photo-oxidation to Mn^{3+}.[14,23]

Figure 1 shows the changes in the six-line absorption EPR lineshape of Mn^{2+} in apo-WOC-PSII in the presence of 10 mM ^{12}C- and ^{13}C-bicarbonate upon illumination at −20 °C, recorded using field-sweep electron spin echo-detected EPR. This concentration of bicarbonate was chosen as it provides full saturation of the bicarbonate site determined from previous photoactivation studies and Mn^{3+} EPR studies.[7,16] In addition to the partially resolved six-line hyperfine pattern arising from Mn^{2+}, the spectrum constitutes an overlapping radical signal at $g = 2.0$ arising from the photooxidized tyrosine radical $Y_D^{\bullet}$. Upon illuminating the sample at −20 °C, the Mn^{2+} intensity decreases in both the ^{12}C- and ^{13}C-bicarbonate samples to an equal extent (Figure 1). This change in the intensity corresponds to photooxidation of

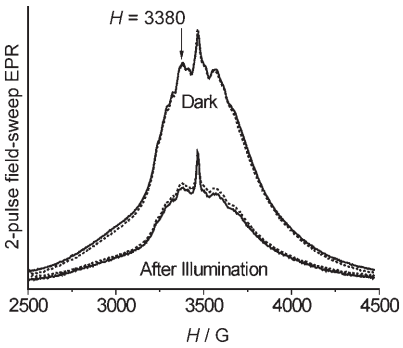

Figure 1. Field-sweep EPR spectra of Mn^{2+} in apo-PSII under dark and after illumination at −20 °C, measured by using the 2-pulse ESE technique. In the presence of 10 mM $NaH^{12}CO_3$ (solid black trace) and 10 mM $NaH^{13}CO_3$ (dashed black line); upper and lower traces shown before (that is, in the dark) and after illumination. The 2-pulse ESEEM was collected at the field position indicated by the arrow. Conditions: interpulse delay $\tau = 400$ ns, pulse $\pi/2 = 16$ ns, temperature 5 K.

[*] Dr. J. Dasgupta, Prof. G. C. Dismukes
7 Hoyt Laboratory
Chemistry Department
Princeton University
Princeton, NJ 08544 (USA)
Fax: (+1) 609-258-3449
E-mail: dismukes@princeton.edu
Dr. A. M. Tyryshkin
Department of Electrical Engineering
Princeton University
Princeton, NJ 08544 (USA)

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ESEEM = electron spin echo envelope modulation.

Supporting information for this article (time-domain 2-pulse ESEEM decays; 2-pulse ESEEM spectra measured at additional magnetic fields; 3-pulse ESEEM spectra of no added and 10 mM ^{13}C-bicarbonate added apo-WOC-PSII samples; and simulation parameters for ^{13}C-ESEEM spectrum) is available on the WWW under http://www.angewandte.org or from the author.

Mn^{2+} bound to the high-affinity PSII site.[15] The EPR spectra in the presence of ^{13}C- and ^{12}C-bicarbonate are identical, indicating that the ^{13}C hyperfine couplings are small and therefore unresolved in the broad EPR linewidth. To spectrally resolve these small couplings, 2-pulse ESEEM experiments were carried out.

The 2-pulse ESEEM was recorded at the magnetic field 3380 G (marked by an arrow in Figure 1) which corresponds to the center of one of the resolved ^{55}Mn hyperfine lines. Measurements for each sample were performed before and after illumination at −20 °C, and a marked reduction of the echo intensity was observed after illumination as explained above (see Figure S1 in the Supporting Information). The difference, "dark-minus-light" cosine FT ESEEM traces recorded for Mn^{2+} in presence of 10 mM ^{12}C- or ^{13}C-bicarbonate are shown in Figure 2 A. A positive peak at 14.4 MHz and a negative peak at 28.8 MHz were observed in all spectra corresponding to the ^{1}H Zeeman frequency and its double frequency, respectively. These peaks arise from weakly coupled ^{1}H nuclei from amino acid residues and water molecules surrounding the photooxidizable Mn^{2+} at the high-affinity PSII site.

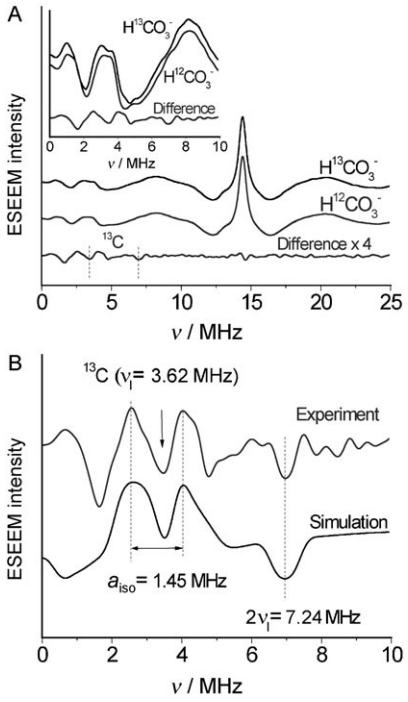

Figure 2. Cosine FT spectra of the 2-pulse ESEEM of the photooxidizable Mn^{2+} bound at the high-affinity site in apo-WOC-PSII at pH 7.5. All spectra were obtained by subtracting the measured ESEEM time-domains before and after illumination at −20 °C. A) The top and middle traces were measured after adding 10 mM ^{13}C- and ^{12}C-bicarbonate, respectively. The bottom trace represents the double difference spectrum of the dark-minus-light ^{13}C- minus ^{12}C-bicarbonate enriched samples, magnified four times. The vertical lines on the bottom trace denotes the ^{13}C Zeeman frequency (ν_I) and its double ($2\nu_I$) at $B_0 = 3380$ G. Inset: the expanded low-frequency region. B) The double difference spectrum from (A) is expanded and compared to the simulated ^{13}C spectrum (see text for simulation parameters). The doublet centered at ^{13}C Zeeman frequency indicates an isotropic coupling $a_{iso} \approx 1.45$ MHz. Experimental conditions: $[Mn^{2+}] = 120$ μM; [apo-WOC-PSII] = 20 μM; the microwave frequency = 9.724 GHz, the magnetic field = 3380 G, and temperature 5 K.

Both "dark-minus-light" difference spectra show nearly identical sets of low-frequency peaks in the range 1–5 MHz (inset to Figure 2 A). These peaks are more clearly resolved in the three-pulse ESEEM spectra, which resolve three peaks at 0.7, 3.7, and 4.9 MHz (see Figure S2 and S3 in the Supporting Information). These three low-frequency peaks are characteristic of ^{14}N nucleus strongly coupled to Mn^{2+}.[17,18] When measured at different parts of the Mn^{2+} EPR spectrum, these peaks show little dependence on the magnetic field strength (Figure S2 in the Supporting Information) which further supports their assignment to ^{14}N nuclei. Detailed spectral simulations will be required to confirm assignments for these peaks and to extract hyperfine and quadrupole ^{14}N parameters. ^{14}N simulations have not been performed. However, we suggest a possible assignment from a histidine or lysine residue based on comparisons with the published spectra of Mn^{2+}–histidine and Mn^{2+}–lysine complexes.[17,18]

Upon closer inspection of the "dark-minus-light" difference spectra (inset Figure 2 A), smaller features can be seen in the low-frequency range in the presence of ^{13}C-bicarbonate ($I = 1/2$) which are absent in the presence of ^{12}C-bicarbonate ($I = 0$). These appear as a small shoulder on the broad positive peak at 3.8 MHz arising from ^{14}N, along with a small negative bump near 7 MHz. The ^{12}C spectrum (middle trace in Figure 2 A) does not show these two features. These additional features are more clearly visible by subtracting the normalized spectra for ^{13}C and ^{12}C-bicarbonate samples. This double difference spectrum is shown in Figure 2 A and also as an expanded trace in Figure 2 B. It eliminates the stronger lines from ^{1}H and ^{14}N nuclei and permits detection of weaker transitions from ^{13}C nuclei. This double difference spectrum gives the ^{13}C–^{12}C difference of the photooxidable Mn^{2+} sites at the high affinity site of PSII. The prominent features in this spectrum are the two positive peaks at 2.55 and 4.03 MHz and the negative peak at 6.96 MHz. The two positive peaks are positioned around the ^{13}C Zeeman frequency ($\nu_I = 3.62$ MHz at 3380 G) and correspond to two ^{13}C nuclear spin transitions within the electron spin manifolds $M_S = \pm 1/2$ of electron spin $S = 5/2$ of Mn^{2+}.[19,20] Splitting between these peaks (ca. 1.4 MHz) gives a measure of isotropic part (a_{iso}) of the ^{13}C hyperfine tensor, and the individual linewidth of each peak (ca. 1 MHz) gives an upper limit for anisotropic part (T) of the ^{13}C hyperfine tensor. The second negative peak $\nu_\sigma =$ 6.96 MHz is the sum combination transition. This peak is slightly shifted to lower frequencies compared to the double ^{13}C Zeeman frequency ($2\nu_I = 7.24$ MHz). Such negative shifts have been theoretically predicted and experimentally verified in the spectra of Mn^{2+} complexes.[20] It indicates the ^{13}C nucleus is interacting with a Mn^{2+} center possessing zero-field splitting (ZFS) and also a specific orientation of the ^{13}C hyperfine tensor with respect to the ZFS tensor.[16,20] This ^{13}C spectrum is a close replica of the spectrum reported for the ^{13}C-labeled complex $[Mn(CO_3)(HCO_3)(OH_2)_3]^-$ in frozen aqua solutions where similar ESEEM peaks were assigned to the ^{13}C nucleus of the bidentate coordinated CO_3^{2-} ligand.[16]

A simulation of the ^{13}C spectrum in Figure 2B was performed by taking into account nonzero ZFS of Mn^{2+}.[20] Large negative ZFS coupling ($D = -375$ G) and the 90° angle between the principal axes of the ZFS and ^{13}C hyperfine tensors had to be assumed to reproduce the significant negative shift of the sum combination peak ν_σ from $2\nu_I$.[16] This large ZFS coupling is much greater than the axial component of the ZFS tensor, $|D| \approx 50$ G of the symmetric Mn^{2+} hexaaqua complex,[21] suggesting a significant ligand-field asymmetry at the Mn^{2+} coordination site in PSII. The simulation provides both the isotropic ($a_{iso} = 1.45$ MHz) and anisotropic ($T = 0.9$ MHz) parts of the ^{13}C hyperfine tensor. These values are of significant magnitude and can only occur for a directly coordinated ligand bound in the first coordination shell of Mn^{2+}. Both values are a close match to those determined for the bidentate coordinated CO_3^{2-} ligand ($r_{^{13}C-Mn} \approx 2.9$ Å) in $[Mn(CO_3)(HCO_3)(OH_2)_3]^-$ complex.[16] We therefore can be confident that ^{13}C-bicarbonate forms a direct ligand to the high-affinity Mn^{2+} center in PSII during the first step of photoassembly of the Mn_4Ca cluster. This site also possesses an unidentified nitrogen-donor ligand, possibly a histidine or a lysine, at this Mn^{2+} site.

Possible candidates for this N-donor ligand can be inferred from the X-ray diffraction structure of an intact cyanobacterial PSII. The only N-donor ligands found within the first coordination shell of the intact Mn_4Ca cluster are histidine residues D1His332 and D1His337. Interestingly, neither of these are close enough to D1Asp170 to bind the same Mn^{2+} ion according to the most recent interpretation of the structural data.[22] D1Asp170 has been proposed to serve as a ligand to Mn^{2+} in the high-affinity site of PSII.[23] While more recent experiments using a histidine residue substituted at this site (D1Asp170His) were ambiguous on this question and left open the possibility that it might not be a direct ligand.[24] Our results are compatible with two possible interpretations. Either Asp170 is not a ligand to the high-affinity Mn^{2+} site, or a structural rearrangement of the Mn ligands would be required in subsequent steps of photoactivation which completes the intact Mn_4Ca coordination sphere. The latter proposal would be consistent with kinetic studies of photoactivation which find a slow conformational rearrangement controls the rate of the second step.[14]

Our work should stimulate further studies on understanding the importance of carbonate concentrations inside cell compartments where biogenesis takes place, as well as the role that bicarbonate is postulated to have played during the evolution of oxygenic photosynthesis.[25] It also raises the level of interest in re-examining spectroscopically the still debated question of participation of bicarbonate in catalyzing the water oxidation reaction[26] within the Mn_4Ca cluster in the intact PSII.[2,6,27]

Experimental Section

Apo-WOC-PSII membranes, depleted of Mn^{2+}, Ca^{2+} and the three extrinsic-proteins, were prepared from spinach PSII membranes as previously described.[7] For EPR experiments, $MnCl_2$ (120 μM) and $NaHCO_3$ (10 mM) were added in the dark to 20 μM of apo-WOC-PSII at pH 7.5. After mixing and 5 min additional incubation, samples of identical volumes were placed in precision quartz EPR tubes prior to freezing at 77 K. ^{13}C-bicarbonate $NaH^{13}CO_3$ was used (99% pure, Cambridge Isotopes Laboratory Inc.). The Mn^{2+}-loaded PSII samples were measured as prepared in the dark and also after illumination at −20 °C, which limits the Mn^{2+} photooxidation step to a single turnover[15] and eliminates secondary reactions at higher temperatures. The difference spectra "dark-minus-light" represents the spectrum of the photooxidizable Mn^{2+} that is bound to the high affinity site in apo-WOC-PSII. Pulsed EPR experiments were performed using an X-band Bruker Elexsys580 spectrometer at temperatures 5–10 K using a liquid helium flow cryostat CF935 (Oxford Inst), using conditions for detection of Mn^{2+}.[16] In all electron spin echo (ESE) experiments the duration of the π/2 and π pulses was set to 16 and 32 ns, respectively. Field-sweep ESE-detected EPR spectra were collected using 2-pulse echo sequence, π/2–τ–π–τ–echo, with fixed interpulse delay $\tau = 400$ ns; the integrated echo pulse intensity was detected while sweeping the magnetic field. For ESEEM experiments the two-pulse echo intensity was measured as a function of τ, using the sequence π/2–τ–π–τ–echo, starting from initial $\tau = 100$ ns and incrementing with 16 ns step. The three-pulse ESEEM experiments were carried out at two τ delays 160 ns and 260 ns with the following sequence: π/2–τ–π/2–T–π/2–τ–echo, where delay T was varied. Prior to Fourier transformation (FT) all ESEEM time-domains were normalized to unity background by fitting the relaxation decays with a smoothing spline function followed by division of the experimental time-domain by the fitted baseline decay.

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