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The Structure of Formaldehyde-Inhibited Xanthine Oxidase Determined by 35 GHz ^{2}H ENDOR Spectroscopy

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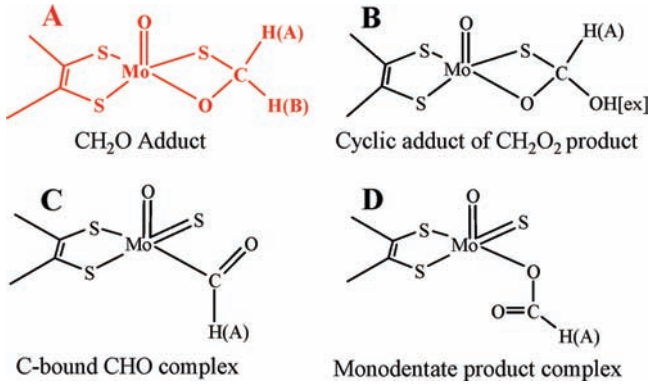
Abstract: The formaldehyde-inhibited Mo(V) state of xanthine oxidase (**I**) has been studied for four decades, yet it has not proven possible to distinguish unequivocally among the several structures proposed for this form. The uniquely large isotropic hyperfine coupling for ^{13}C from CH_2O led to the intriguing suggestion of a direct Mo−C bond for the active site of **I**. This suggestion was supported by the recent crystal structures of glycol- and glycerol-inhibited forms of aldehyde oxidoreductase, a member of the xanthine oxidase family. ^{1}H and ^{2}H ENDOR spectra of **I**($C^{1,2}H_2O$) in H_2O/D_2O buffer now have unambiguously revealed that the active-site structure of **I** contains a CH_2O adduct of Mo(V) in the form of a four-membered ring with S and O linking the C to Mo and have ruled out a direct Mo−C bond. Density functional theory computations are consistent with this conclusion. We interpret the large ^{13}C coupling as resulting from a "transannular hyperfine interaction".

Xanthine oxidase is a molybdoenzyme that catalyzes the oxidative hydroxylation of a variety of heterocyclic and aldehyde substrates, including the physiological substrates hypoxanthine and xanthine.[1] Of the large number of EPR-active Mo(V) intermediates exhibited by this enzyme,[2] one of the most intriguing is the CH_2O-inhibited Mo(V) form (**I**) first described by Bray and co-workers.[3] Its most remarkable feature, which was revealed by Howes and co-workers,[4,5] is the presence of a carbon derived from CH_2O that EPR and ENDOR spectroscopies show to have a uniquely large ^{13}C isotropic hyperfine coupling, $a_{iso}(^{13}C) \approx 43.0$ MHz. This value contrasts with the 5-fold smaller coupling for the Mo−O−^{13}C of the "2-hydroxy-6-methyl-purine (HMP) very rapid" form (a_{iso} = 7.9 MHz).[6] Although **I** has been studied for 40 years, it has not proven possible to distinguish unequivocally among the viable candidates for its structure (Scheme 1).[5,7−13]

The large isotropic ^{13}C hyperfine coupling for **I** led Howes and co-workers to the intriguing suggestion that it contains a CHO fragment with a direct Mo−C bond (**C** in Scheme 1).[4,5] This suggestion recently received support from the crystal structures of glycol- and glycerol-inhibited forms of aldehyde oxidoreductase (AOR), a member of the xanthine oxidase family.[7] These structures exhibit Mo−C bond distances of 2.36 and 2.72 Å, respectively, the former in particular being suggestive of direct Mo−C bonding interactions. In contrast, analysis of the smaller ^{13}C coupling for a substrate-derived species bound to Mo(V) of the "very rapid" state of xanthine oxidase indicated that there was no direct Mo−C bond,[6] and this was confirmed by recent crystal structures for that intermediate.[14,15]

Scheme 1



We here report that ^{1}H and ^{2}H ENDOR spectra of **I**($C^{1,2}H_2O$) prepared[16] in H_2O/D_2O buffer rule out all of the models proposed for the active-site structure of **I** (Scheme 1) except for model **A**, a four-membered cyclic adduct of CH_2O with S and O linking the C to Mo. Density functional theory (DFT) calculations are consistent with the ENDOR finding that **A** rather than the direct Mo−C bond of **C** represents the structure for the active site of **I**.

The reported X-band EPR spectrum of **I**($^{13}C^1H_2O$) in H_2O shows a doublet splitting from the ^{13}C nucleus, with each line further split into a doublet from a single proton derived from CH_2O.[4] The 35 GHz echo-detected EPR spectrum of **I**($^{12,13}C^1H_2O$) in H_2O shows the ^{13}C doublet from $^{13}C^1H_2O$, but the ^{1}H splitting is not observed (Figure S1).[17,18] The EPR spectrum of **I**($^{12,13}C^1H_2O$) further shows hyperfine splitting from ^{95,97}Mo (natural abundances: ^{95}Mo, 15.9%; ^{97}Mo, 9.6%). The g_1 and g_3 splittings are observable in both the X- and Q-band spectra; those associated with g_2 are resolved only in the Q band spectrum (Figure S1). Simulations of the EPR spectra gave principal values of the **g** tensor and values of the components of **A** for the ^{13}C of CH_2O that agree with those of Howes and co-workers: **g** = [1.988, 1.974, 1.948], **A**(^{13}C) = [51.5, 40, 40] MHz, a_{iso} = 43.8 MHz.

Figure 1 presents 35 GHz Davies ^{1}H and ^{95,97}Mo (top) and Mims ^{2}H (bottom) ENDOR spectra collected for **I**($^{12}C^{1,2}H_2O$) in H_2O/D_2O near g_2, the magnetic field corresponding to the maximum EPR intensity. The ^{1}H Davies spectra of **I**(C^1H_2O) in H_2O and D_2O are essentially the same (traces **A**), both exhibiting an ^{1}H doublet centered at the ^{1}H nuclear Larmor frequency and split by $A \approx 13$ MHz. This doublet is absent in the spectrum of **I**(C^2H_2O) in H_2O (trace **B**) and thus is associated with a proton, ^1H_A, derived from CH_2O. The ^{1}H doublet in Figure 1 rides on ^{95,97}Mo ENDOR signals and is more clearly seen in the difference spectrum **A**−**B** obtained by subtraction of the spectrum of **I**(C^2H_2O) (**B**) from that of **I**(C^1H_2O) (**A**).

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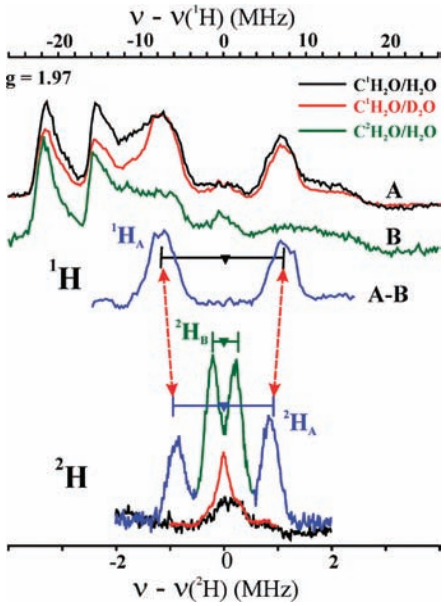

Figure 1. (top) Davies ^{1}H and $^{95,97}Mo$ (**A**, **B**) ENDOR spectra (35 GHz) of **I**($C^{1,2}H_2O$) in H_2O and D_2O buffers. (bottom) Mims ^{2}H ENDOR spectra (35 GHz) of **I**($C^{2}H_2O$) in H_2O (green/blue), **I**($C^{1}H_2O$) in H_2O (black) and D_2O (red) buffer. Horizontal bars indicate hyperfine splittings for $^{1,2}H_{A,B}$ nonexchangeable protons. Conditions: $g = 1.97$; $T = 2$ K. Davies: π-pulse = 80 ns, τ = 600 ns, repetition time = 50 ms, 34.84 GHz. Mims ^{2}H: $^{\pi}/_{2}$ pulse = 50 ns, τ = 800 ns, repetition time = 50 ms, 34.87 GHz.

The $^{1}H_A$ doublet is lost and is replaced by a corresponding $^{2}H_A$ signal when $C^{2}H_2O$ is the substrate. ^{2}H ENDOR spectra of **I**($C^{2}H_2O$) in H_2O by both Davies (not shown) and Mims (Figure 1, lower) techniques exhibit a $^{2}H_A$ doublet without resolved quadrupole splitting. The hyperfine interaction, **A**($^{2}H_A$), corresponds to a smaller value of **A**($^{1}H_A$) than that seen directly in the ^{1}H ENDOR spectrum of **I**($C^{1}H_2O$) in H_2O/D_2O because of a substantial isotope effect on the hyperfine couplings.[19,20] Most importantly, the ^{2}H Mims spectrum of **I**($C^{2}H_2O$) in H_2O shows doublets from *both* deuterons derived from the $C^{2}H_2O$ (Figure 1), a second doublet, denoted $^{2}H_B$, plus that from $^{2}H_A$.

To test for protons exchangeable with solvent, Mims ^{2}H ENDOR spectra were collected for **I**($C^{1}H_2O$) in D_2O and **I**($C^{2}H_2O$) in H_2O at g_2 (Figure 1, bottom). The nearly featureless ^{2}H spectrum of **I**($C^{1}H_2O$) in D_2O shows clearly that there are no exchangeable protons in the active site of **I**.

To confirm that the Mims ^{2}H spectrum is not significantly distorted by suppression "holes" associated with this technique[21] and to determine the ^{2}H hyperfine and quadrupolar tensors, we collected a complete 2D field−frequency ^{2}H ENDOR pattern of spectra acquired at numerous magnetic fields across the EPR envelope and simulated this pattern with inclusion of the suppression effects (Figure S2 in the Supporting Information).[22] At magnetic fields near g_1, the spectra show the $^{2}H_A$ and $^{2}H_B$ doublets centered at the ^{2}H Larmor frequency and split by $A \approx 1.8$ and 0.4 MHz, respectively. The ^{2}H ENDOR pattern does not change significantly as the field is moved toward g_2 and g_3, except in intensity, indicating that the hyperfine couplings of $^{2}H_A$ and $^{2}H_B$ are largely isotropic. The 2D pattern is well-simulated by the 1:1 summation of simulations of $^{2}H_A$ and $^{2}H_B$, with **A**($^{2}H_A$) = [1.8, 1.8, 1.9] MHz and **A**($^{2}H_B$) = [0.44, 0.4, 0.39] MHz. Neither ^{2}H exhibits quadrupolar splitting ($I = 1$) because of the large ENDOR line widths. As noted above for the g_2 spectrum, the tensor obtained from simulations of the $^{2}H_A$ ENDOR pattern only approximately matches that obtained by fitting a 2D ^{1}H Davies ENDOR pattern for $^{1}H_A$ because of an isotope effect on the hyperfine couplings.[19,20]

*The finding that both aldehydic protons of CH_2O are retained in **I** and are nonexchangeably hyperfine-coupled to the Mo(V) of **I** rules out all of the candidates for the active-site structure of **I** shown in Scheme 1 except the cyclic CH_2O adduct, **A**.*

DFT calculations (SI) are consistent with experiment, yielding hyperfine tensors for ^{13}C, $^{2}H_A$, and $^{2}H_B$ of CH_2O in **A** that all are in satisfactory agreement with the experimentally observed values: $a_{iso}(^{13}C) \approx 47.9$ MHz, **A**(^{13}C) = [53.6, 45.4, 44.6] MHz; **A**($^{2}H_A$) = [4.0, 3.3, 3.0] MHz; **A**($^{2}H_B$) = [0.38, −0.67, −0.72] MHz. Most importantly, the carbon-bound CHO complex, model **C**, which is already ruled out by the absence of H_B, is calculated to have almost 3-fold and 5-fold smaller ^{13}C and $^{1}H_A$ hyperfine couplings, respectively: $a_{iso}(^{13}C) \approx 16.1$ MHz, **A**(^{13}C) = [23.2, 13.4, 11.7] MHz; **A**($^{2}H_A$) = [0.51, −0.43, −0.66] MHz.

Why is the ^{13}C coupling so much larger for the carbon bound in the four-membered ring of structure **A**, and not directly coordinated to Mo(V), than it would be for a carbon directly bonded to the metal ion in **C**? We interpret the large coupling in **A** as resulting from a "transannular hyperfine interaction".[23,24] The carbon is in line with a lobe of the half-occupied Mo(d_{xy}) orbital, and this allows overlap between Mo(d_{xy}) and orbitals of carbon; the large a_{iso} for ^{13}C corresponds to only ∼1.2% spin density in a carbon 2s orbital. This phenonmenon was first observed for a phosphorus atom that formed part of four-membered cyclic structures of Mo(V) and V(IV) with dialkyl/aryldithiophosphinato ligands.[23,24] A weaker hyperfine coupling was observed when the ^{31}P is bound to the metal ion directly through a monodentate M−O−P linkage,[25] and the same is true for the Mo−O−^{13}C of the "very rapid" intermediate.[6] Transannular effects may have relevance to a recent proposal of a direct Fe−C bond based on a large ^{13}C coupling.[26]

Finally, regarding the use of distances in the X-ray structure to infer a direct Mo−C bond, we note that the DFT-optimized cyclic structure **A** has a Mo−C distance of 2.76 Å, which is the same as that found by X-ray diffraction for the glycerol-inhibited form of AOR.[7]

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Supporting Information Available: One EPR figure, one ENDOR figure, and DFT calculation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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