# Proton ENDOR Identification of Bridging Hydroxide Ligands in Mixed-Valent Diiron Centers of Proteins: Methane Monooxygenase and Semimet Azidohemerythrin

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The hydroxylase component of methane monooxygenase (MMO) belongs to a recently identified class of non-heme iron carboxylate proteins including hemerythrin, ribonucleotide reductase, and purple acid phosphatase, all of which contain a diiron center at their active sites.[2,3] Physical studies to determine the structure of this center in the hydroxylase from *Methylococcus capsulatus* (Bath) have been carried out in our laboratory and elsewhere.[4-6] Results from EXAFS,[4,5] Mössbauer,[4] and EPR[4,6,7] measurements provide indirect evidence for a hydroxo, alkoxo, or monodentate carboxylato bridge linking the two iron atoms in the oxidized ($H_{ox}$, Fe(III)Fe(III)) and mixed-valent ($H_{mv}$, Fe(II)Fe(III)) forms of the protein. Similar observations and conclusions were reached from studies of the related MMO hydroxylase from *M. trichosporium* OB3b.[8,9] Given the likely functional dependence of the protein active site on the nature of the bridging ligand, we have carried out 35-GHz electron nuclear double resonance (ENDOR)[10] spectroscopic studies of $H_{mv}$ from *M. capsulatus* (Bath) in pursuit of this information.

In the present communication we report the results of proton ENDOR investigations that identify a bridging hydroxide ligand in $H_{mv}$ and further reveal the existence of a solvent-derived ($H_2O$ or $OH^-$) terminal ligand coordinated to the dinuclear center. The former assignment is based on a striking spectral similarity between the $H_{mv}$ proton ENDOR spectrum and that of semimet azidohemerythrin ($HrN_3$), for which a large body of evidence strongly suggests the presence of a hydroxo-bridged mixed-valent diiron moiety.[11-14] In fact, the present observation of the ENDOR signal for semimet $HrN_3$ may be taken as additional evidence for the $\{Fe_2(OH)\}^{4+}$ core in this protein and affords a spectroscopic signature of this unit that should be of general utility.

The mixed-valent forms of the MMO hydroxylase and $HrN_3$ have rhombic EPR signals with $g_{av} < 2$, characteristic of the $S = {}^1/_2$ state of antiferromagnetically exchange-coupled, valence-localized dinuclear iron centers. In Figure 1 are presented proton ENDOR patterns for $H_{mv}$ and semimet $HrN_3$, each in both $H_2O$ and $D_2O$ buffer.[15,16] Data recorded at two different $g$-values for each protein have been extracted from numerous spectra taken across the EPR envelope in order to evaluate the relative anisotropies of the proton hyperfine interaction tensors.[10]

Comparison of the proton ENDOR spectra of $H_{mv}$ in $H_2O$ and $D_2O$ shows that the resonances can be grouped into three classes. The first class consists mainly of nonexchangeable protons that persist in the $D_2O$ sample and have small hyperfine coupling values (Figure 1, $A_1 \leq 4$ MHz). They arise primarily from constitutive protons of endogenous Fe ligands and are more highly resolved in spectra taken under other conditions (data not shown). The second class, marked by the double arrow in Figure 1, is exchangeable in $D_2O$ buffer.[19] This class has a hyperfine coupling $A_2 \sim 8$ MHz and is nearly isotropic; that is, its resonance position changes little at different $g$-values. Such properties are reminiscent of those previously observed for $H_2O$ and $OH^-$ bound to an iron atom of the $[4Fe\text{–}4S]^+$ cluster of aconitase.[21]

The third class of protons, marked with a brace in Figure 1, also exchanges with $D_2O$ and has quite remarkable properties. It gives rise to one doublet with a hyperfine coupling of ~14 MHz at $g_1$, and as the magnetic field is increased, it splits into three doublets which, at $g_2$, have achieved a maximum coupling of $A_3 \sim 30$ MHz. Preliminary simulations indicate that these multiple features can be assigned to a single proton where the resonances at field values away from the edges of the EPR envelope are split by hyperfine anisotropy.[10] The characteristics of the hyperfine tensor, including the extent of its anisotropy (14–30 MHz), the size of the maximum splitting, and the isotropic

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(15) Methane monooxygenase hydroxylase with specific activity between 200 and 250 mU/mg with propylene was isolated and purified as described[4] and concentrated to ~1 mM in 25 mM MOPS (MOPS = *N*-morpholinopropanesulfonic acid), pH 7. $H_{mv}$ was prepared by anaerobic reduction with dithionite (0.5 mM) in the presence of mediators, 1 mM each of phenazine methosulfate, methylene blue, and potassium indigosulfonate. This procedure typically converts ≥50% of the protein to $H_{mv}$. Deuterium exchange was performed over a 15-h period by washing protein adsorbed on a Q-Sepharose column with $D_2O$ buffer prior to reduction. Semimet $HrN_3$ in 50 mM Tris acetate, pH 8, was prepared from *Phascolopsis gouldii* met Hr (~5 mM) by anaerobic reduction using dithionite followed by addition of an excess of $NaN_3$. For deuterated samples, met Hr was exchanged into deuterated buffer by successive dilution and concentration over a period of 10–24 h prior to reduction to the semimet form. The EPR spectra of these samples were identical to those published previously.
(16) ENDOR data were collected with a 35-GHz CW ENDOR spectrometer of local design operating in dispersion mode.[17,18] The ENDOR pattern for a proton of type i consists of a doublet split by the hyperfine interaction $A_i$ and centered at the proton Larmor frequency, ~55 MHz for our measurements. Of note for 35-GHz ENDOR is that the higher frequency partner of the doublet is typically more intense, and so the proton ENDOR patterns may appear quite asymmetric in intensity.[10]
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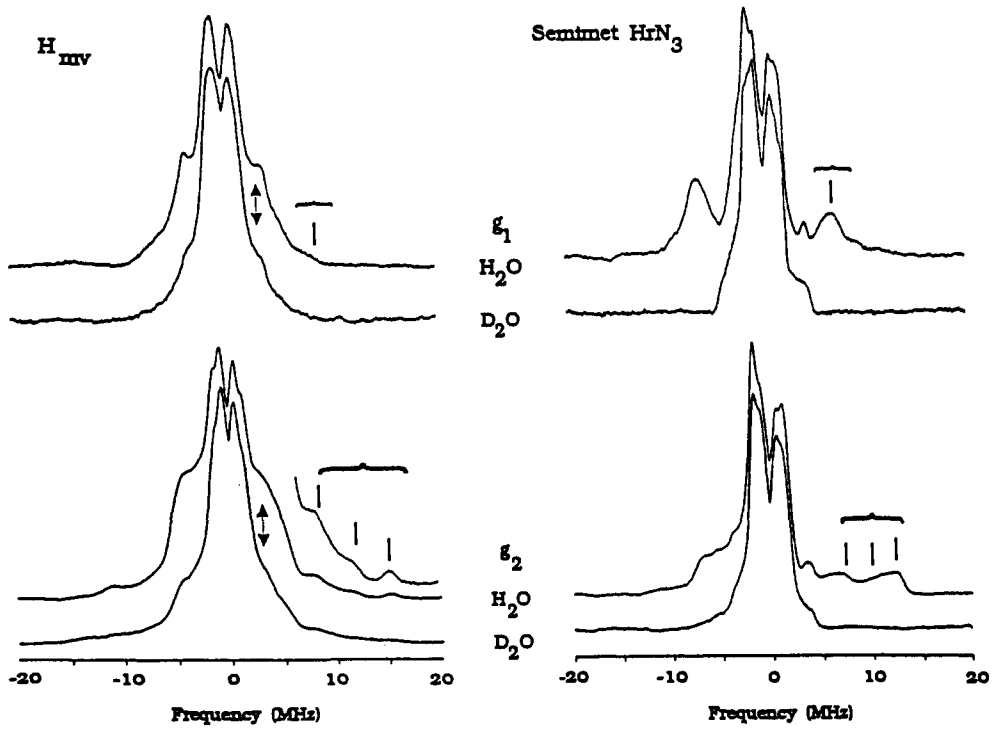


**Figure 1.** Proton ENDOR spectra at $g_1$ (upper) and $g_2$ (lower) of mixed-valent MMO hydroxylase (left) and semimet hemerythrin azide (right) with and without exchange into $D_2O$. (Left) The proton resonances for $H_{mv}$ can be grouped into three classes. The first has small hyperfine coupling values ($A_1 < 4$ MHz). The second, marked by the double arrow, gives rise to larger couplings, $A_2 \sim 8$ MHz, and is exchangeable in $D_2O$. The third class of protons, marked by a brace and vertical lines, is also exchangeable in $D_2O$. Spectrometer conditions for dispersion mode EPR–ENDOR were as follows: 35.2-GHz microwave frequency, 3.2 mW, 2 K, 30-W rf, 2-G modulation amplitude, 2 MHz/s scan rate, 12 950 G ($g_1$), 13 400 G ($g_2$), ~100 scans each. (Right) The proton ENDOR spectrum of semimet $HrN_3$ consists of a group of resonances clustered narrowly around the Larmor frequency ($A < 4$ MHz) and a second set of resonances, marked by a brace and vertical lines, at larger hyperfine coupling values. Spectrometer conditions were as above with the following exceptions: 1 MHz/s scan rate, 13 100 G ($g_1$), 13 800 G ($g_2$), ~50 scans each.

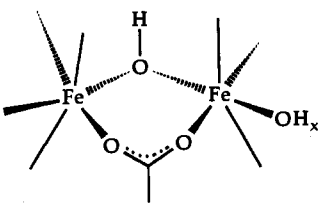


**Figure 2.** Sketch of the diiron center in $H_{mv}$, the hydroxo bridge of which was deduced from the present ENDOR study.

coupling (~24 MHz), are incompatible with an exchangeable proton from a terminal ligand, for example, histidine.[22] They are also incompatible with a proton contributed by a group that is hydrogen-bonded to an Fe ligand.[23] Instead, they suggest an assignment as the H atom of a hydroxide bridge.

These assignments are confirmed by comparison with the proton ENDOR spectra of semimet $HrN_3$, which is known to contain the $\{Fe_2(OH)\}^{4+}$ core with the terminal ligation set $(His)_5(N_3^-)$.[11-14] The spectra for semimet $HrN_3$ display ENDOR signals that arise from class 1 protons (Figure 1, right), but no signals are evident from exchangeable class 2 protons. Because there is no terminal $H_2O$ or $OH^-$ ligand in semimet $HrN_3$, this result strongly supports the assignment of the class 2 protons for $H_{mv}$ as $H_2O$ or $OH^-$ bound to Fe at a terminal position. Most importantly, semimet $HrN_3$ also displays a set of resonances that are very anisotropic, have a hyperfine coupling of ~12 MHz at $g_1$ extending to a maximum coupling of ~28 MHz at $g_2$, and exchange in $D_2O$ (Figure 1, right). The striking correspondence between these class 3 proton signals and those discussed above for $H_{mv}$ leaves no doubt that both signals have the same chemical origin.[24] In semimet $HrN_3$ such a signal can only be assigned to the hydroxide bridge, and therefore the same assignment must obtain for $H_{mv}$. The model for the dinuclear Fe center of $H_{mv}$ that accounts for these results is depicted in Figure 2.

EXAFS and other physical data[4-9] for the diiron(III) form of MMO ($H_{ox}$) reveal the same Fe···Fe distance as in $H_{mv}$ and no oxo bridge. It therefore seems likely that a hydroxide bridge also links the two ferric ions in $H_{ox}$. Such a conclusion would require that the $pK_a$ value of the $\{Fe_2(OH)\}^{+5}$ moiety in $H_{ox}$ be higher than that of metHr.

In conclusion, we have identified a strong, anisotropic ENDOR proton signal arising from the hydroxide bridge of the mixed-valent forms of the dinuclear iron centers in methane monooxygenase hydroxylase and azidohemerythrin. This result is consistent with earlier suggestions of $RO^-$ (R = H or other) bridges in both proteins. The direct observation by 35-GHz ENDOR of the proton involved in the hydroxo bridge in these diiron centers provides a spectroscopic signature of potential utility in identifying such units in other systems.

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(22) For an antiferromagnetically exchange-coupled high-spin $Fe^{III}$–$Fe^{II}$ pair, the maximal hyperfine coupling ($A$) can be related to that observed in a mononuclear system ($a$) by the relation $A \leq \sim 7/3a$.[20] The hyperfine couplings from both exchangeable and nonexchangeable protons on histidine bound to the high-spin ferric ion of myoglobin have been determined [Mulks, C. F.; Scholes, C. P.; Dickinson, L. C.; Lapidot, A. *J. Am. Chem. Soc.* **1979**, *101*, 1645–1653] to be $< \sim 2$ MHz, resulting in an expected $A \leq \sim 5$ MHz for these protons in the exchange-coupled system of $H_{mv}$.

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(24) Accessibility of solvent to the oxidized diiron center of MMO appears to be lower than that for Hr. The rigorous H/D exchange conditions employed here[15] leave no $^1H$ signal from residual $OH^-$ bridge in semimet $HrN_3$, but careful inspection of Figure 1 shows a weak residual signal for $H_{mv}$.