J. Am. Chem. Soc. 1993, 115, 8881-8882

Pulsed EPR Studies of Mixed Valent [Fe(II)Fe(III)] Forms of Hemerythrin and Methane Monooxygenase: Evidence for a Hydroxide Bridge

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Received June 17, 1993

Hemerythrin (Hr) and the hydroxylase component of methane monooxygenase (MMOH) both contain non-heme binuclear iron clusters. These sites serve different functions: in Hr they reversibly bind dioxygen for transport,[1] while in MMOH they activate dioxygen for hydroxylation of hydrocarbons.[2] From spectroscopy[3] and crystallography[4] it is known that the met-[Fe(III)Fe(III)] sites of Hr have an oxo bridge which dominates the bonding interaction between the two ferric sites and leads to a large antiferromagnetic (AF) coupling ($J \approx -135$ cm^{-1}) producing an EPR inactive singlet ground state.[5] In contrast, there is no evidence for an oxo bridge in met MMOH[6] despite the fact that this is an AF coupled ($J \approx -7.5$ cm^{-1}) binuclear iron site.[7] The bridging ligand in MMOH responsible for the AF coupling might be a hydroxide or a functional group associated with a protein residue.

Chemical and spectroscopic studies[8] on $^1/_2$-met$_r$ (produced by one-electron reduction of the met site),[9,10] $^1/_2$-met$_o$ (produced by one-electron oxidation of the fully reduced (or deoxy) [Fe(II)-Fe(II)] site[9,10]), and 1/2-metN$_3^-$ have shown that all three derivatives have a hydroxide bridge. For 1/2-metN$_3^-$, the exogenous azide has replaced an additional terminal OH$^-$ which is present in $^1/_2$-met$_r$ but not in $^1/_2$-met$_o$ Hr.[8] The OH$^-$ bridge in these $^1/_2$-met (or semi-met) [Fe(II)Fe(III)] derivatives leads to AF coupling of Fe(III) $S = {}^5/_2$ with Fe(II) $S = 2$ to produce an $S = {}^1/_2$ ground state which is EPR detectable. In this communication, we report the first pulsed EPR (ESEEM and Davies ENDOR) studies on these three $^1/_2$-met derivatives to define the characteristic hyperfine couplings for the bridging and terminal hydroxide protons. We have used these spectral features to establish the presence of both a bridging and a terminal hydroxide in $^1/_2$-met MMOH. The $^1/_2$-met derivatives were prepared as described previously,[8,11] except that metMMOH was incubated for 25 h at 4 °C in H$_2$O or D$_2$O before reduction. The MMOH spectra shown were recorded in buffer with 2% DMSO, but similar results were obtained in the absence of DMSO.

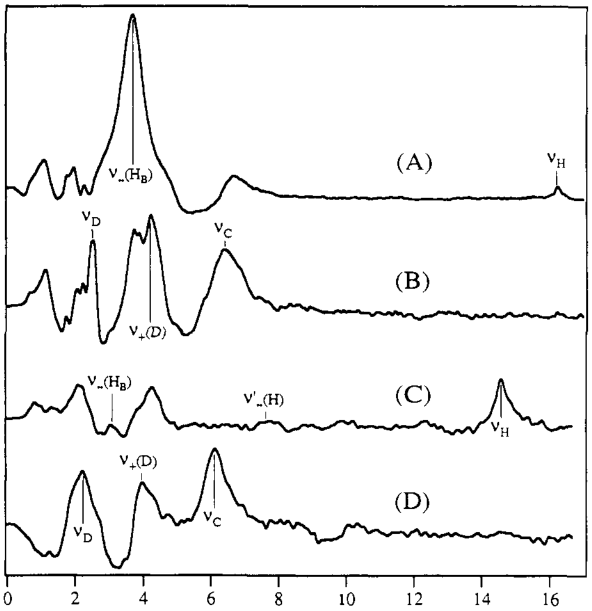

Figure 1. ESEEM spectra recorded with the stimulated echo pulse sequence with $\tau = 0.18$ μs for $^1/_2$-met$_o$ hemerythrin at $g = 1.70$ in (A) H$_2$O and (B) D$_2$O and for $^1/_2$-met MMOH at $g = 1.93$ in (C) H$_2$O and (D) D$_2$O. For A,B: 9.10 GHz; 1.67 K. For C,D: 9.265 GHz; 4.25 K.

ESEEM and Davies ENDOR spectra of $^1/_2$-met$_o$ Hr in H$_2$O and D$_2$O recorded at $g = 1.7$ are shown in Figures 1A,B and 2A,B, respectively. ESEEM and ENDOR spectroscopies are complementary techniques for measuring nuclear hyperfine couplings not resolved in the EPR spectrum.[12] A prominant ESEEM line at 3.7 MHz in Figure 1A, denoted by $\nu_-(H_B)$ is not observed in Figure 1B and thus arises from an exchangeable proton. The broad peak at 28 MHz in the Davies ENDOR spectrum, Figure 2A, which is also exchangeable, Figure 2B, is the partner line, $\nu_+(H_B)$. The hyperfine coupling, $|A(H_B)| = 25$ MHz, is given by $\nu_+(H_B) - \nu_-(H_B)$.

These assignments are corroborated by the observation of new ESEEM lines at $\nu_\pm(D)$, Figure 1B, as predicted from $|A(H_B)|$. These line assignments are further supported by the observation of the strong combination frequency[13] line at $\approx$6.5 MHz, denoted by ν_C in Figure 1B, which is assigned to the combination of the $\nu_+(D)$ and the ^{14}N ESEEM lines (from coordinated histidines) at 2.1 MHz. An alternative assignment for ν_C in Figures 1B and 1D is to a second ^{2}H fundamental, $\nu_+'(D)$. This would correspond to a ^{2}H couling of $\approx$8 MHz, yielding a ^{1}H coupling of $\approx$52 MHz. No evidence for such a large coupling was observed in ENDOR spectra. These combined ESEEM and Davies ENDOR experiments establish a hyperfine coupling of 25 MHz at $g = 1.7$ for an exchangeable proton in $^1/_2$-met$_o$ Hr.

Combined ESEEM and Davies ENDOR experiments have also been extended to measurements at both $g_\perp$ and $g_{//}$ for all three derivatives of $^1/_2$-met$_o$ Hr. The results for the anisotropic hyperfine couplings, $|A(H_B)|$, for the exchangeable proton associated with the bridging OH$^-$ in the $^1/_2$-met Hr derivatives are summarized in Table I. In addition, an exchangeable proton $|A(H_T)| \approx 8$ MHz and nearly isotropic coupling is observed for $^1/_2$-met$_r$ but not for the other two $^1/_2$-met Hr derivatives. Thus these results also establish the hyperfine signature of the terminal hydroxide proton in the mixed-valent Hr site.

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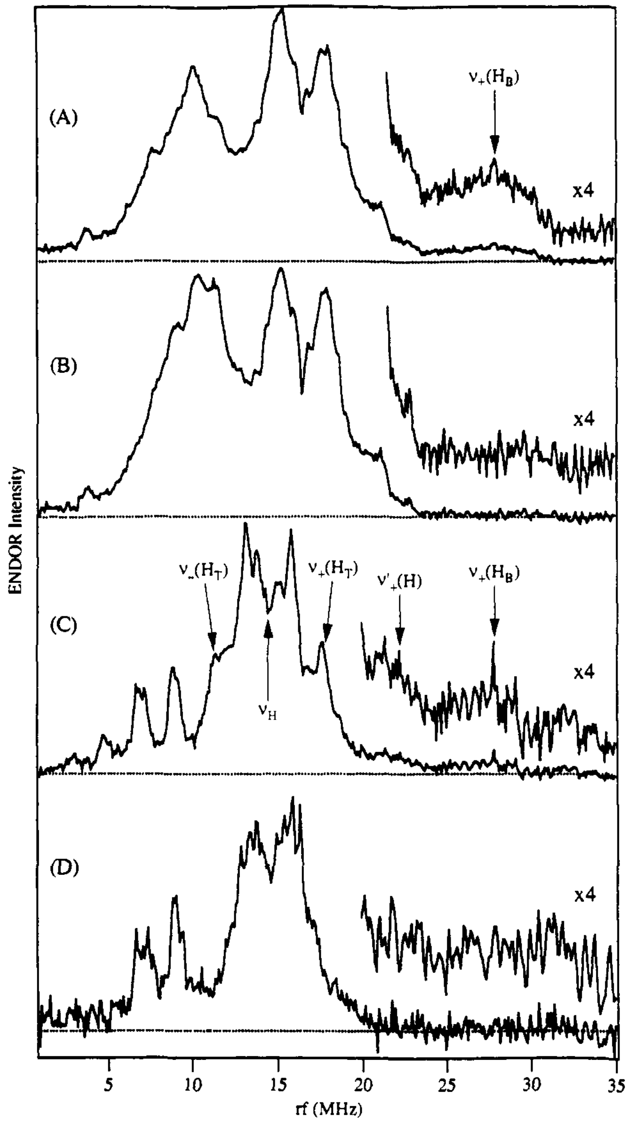

Figure 2. Davies ENDOR recorded at $g = 1.70$ for hemerythrin $^1/_2$-met$_o$ in (A) H_2O and in (B) D_2O and for MMOH recorded at $g = 1.94$ in (C) H_2O and in (D) D_2O. Microwave pulse widths 100, 50, 100 ns; 9.265 GHz; rf pulse 6 μs;

Table I. Summary of Hyperfine Couplings

sample	g-value	$A(H_B)$ (MHz)
$^1/_2$-met$_o$	1.70	25
$^1/_2$-met$_o$	1.92	14
$^1/_2$-metN_3^-	1.68	31
$^1/_2$-metN_3^-	1.90	13.5
$^1/_2$-met$_r$	1.70	23
$^1/_2$-met$_r$	1.92	17
MMO	1.94 (g_1)	23
MMO	1.86 (g_2)	16
MMO	1.76 (g_3)	13

From Table I, a large anisotropic hyperfine coupling is associated with the bridging OH^- proton in the 1/2-met Hr derivatives with $|A_{max}|$ along $g_\perp$ in $^1/_2$-met$_o$, while for both $^1/_2$-met$_r$ and $^1/_2$-metN_3^-, $|A_{max}|$ is along $g_{//}$. The orientation of the g-tensor is determined by the zero-field splitting, D, of the ferrous site. The magnitude and anisotropy of the g-tensor are determined by D/J, where J is the exchange coupling.[14] This can also contribute to the anisotropy of the hyperfine coupling. A larger anisotropy is expected when $J \approx D$. All $^1/_2$-met Hr derivatives have $J \approx -8$ cm^{-1}.[8,10,15] For $^1/_2$-metN_3^- $D \approx -6$ cm^{-1}, and for $^1/_2$-met$_r$, $D \approx -7$ cm^{-1} but for $^1/_2$-met$_o$ $D \approx +3$ cm^{-1}.[8] The change in the sign of D is consistent with the change in orientation of A_{max} from $g_{//}$ to $g_\perp$ between the $^1/_2$-met derivatives discussed above. An additional contribution to the hyperfine anisotropy is the dipole interaction between the proton on the hydroxide bridge and the two iron sites.

ESEEM spectra recorded at $g = 1.94$ for $^1/_2$-met MMOH in H_2O and in D_2O are shown in Figure 1C and 1D, respectively. The ESEEM lines at 3.1 and 7.7 MHz in Figure 1C denoted by $\nu_-(H_B)$ and $\nu_-'(H)$ are assigned to protons with $|A(H_B)| = 23$ Hz and $|A(H)| = 13$ MHz, respectively. These line assignments are again supported by the observation of the strong combination frequency line at $\approx$6.1 MHz, denoted by ν_C in Figure 1D, which arises from the $\nu_+(D)$ and the ^{14}N ESEEM lines (from coordinated histidines). Furthermore, the partner lines denoted by $\nu_+(H_B)$ and $\nu_+'(H)$ are observed in the Davies ENDOR spectrum, Figure 2C, recorded at the same g value. These protons as well as a proton with $|A(H_T)| = 8$ MHz are observed to be exchangeable, as is evident by comparing Figures 2C and 2D. In an earlier ENDOR study[11] protons with $|A| \approx 8$ MHz at g_1 and 13 MHz at g_3 were observed but could not be assigned since they did not appear to exchange after incubation for 8 h in D_2O.

Since the magnitude and anisotropy of the hyperfine constant characteristic of the bridging OH^- observed in the $^1/_2$-met Hrs is also observed for $^1/_2$-met MMOH, a OH^- bridge is likely also present in $^1/_2$-met MMOH. Using the $^1/_2$-met Hr data presented here as a reference, we can now also assign the previously reported[13] anisotropic ^2H coupling observed in ESEEM studies of uteroferrin to a hydroxide bridging ligand at the Fe(II)/Fe(III) site. The observation of an exchangeable proton with an isotropic hyperfine coupling of the same magnitude as the terminal hydroxide proton in $^1/_2$-met$_r$ Hr further suggests that the ferric iron in $^1/_2$-met MMOH is also coordinated by a hydroxide ligand. A second exchangeable proton in MMOH with $|A_H| \approx 13$ MHz at $g = 1.94$, denoted by $\nu_+'(H)$ in Figure 2, suggests that the $^1/_2$-met MMOH site may exist in two forms. This has also been suggested in EPR[11] and resonance Raman[16] studies.

According to EXAFS,[6] integer spin EPR,[7] and Mössbauer[7] studies, met MMOH does not contain an oxo bridge. Oxidation of $^1/_2$-met MMOH to the met level thus likely results in retention of an OH^- bridge. This is in contrast to the behavior of Hr, where the OH^- bridge in the $^1/_2$-met site becomes an oxo bridge upon oxidation to the met site. The origin of this difference in apparent pK_as of the hydroxide bridge proton may relate to the inductive effect of more oxygen ligation in MMOH, consistent with the difference in the ENDOR spectra (Figure 2) of $^1/_2$-met Hr and MMOH, which indicate that MMOH is coordinated by fewer nitrogen ligands than Hr.

Acknowledgment. E.I.S. thanks NSF Grant MCB9019752, and J.D.L. thanks NIH Grant GM40466 for support.

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