35 GHz ENDOR Characterization of the “Very Rapid” Signal of Xanthine Oxidase Reacted with 2-Hydroxy-6-methylpurine (^{13}C8): Evidence against Direct Mo−C8 Interaction

Palanichamy Manikandan,[†] **Eun-Young Choi,**[‡,§] **Russ Hille,***[,‡] **and Brian M. Hoffman***[,†]

Contribution from the Department of Chemistry, Northwestern University, Evanston, Ilinois 60208 and Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210

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Abstract: Xanthine oxidase is a molybdenum-containing enzyme that catalyzes the hydroxylation of xanthine and a wide variety of other aromatic heterocycles. In the course of the reaction with xanthine and substrates such as 2-hydroxy-6-methylpurine (HMP), the enzyme gives rise to a Mo(V) EPR signal, denoted “very rapid”, that arises from an authentic catalytic intermediate. The two alternative catalytic mechanisms proposed for this enzyme differ critically in whether the distance between Mo and C8 of the purine nucleus in this intermediate is short enough to admit a direct bonding interaction. To examine this distance, we have performed ^{13}C ENDOR measurements of the “very rapid” EPR signal generated by xanthine oxidase during reaction with ^{13}C8-HMP. The resulting ^{13}C8 hyperfine tensor, $\mathbf{A} = [10.2(1), 7.0(1), 6.5(1)]$ MHz, is discussed in the framework of a detailed consideration of factors involved in extracting metrical parameters from an anisotropic hyperfine interaction composed of contributions from multiple sources, in particular, the effect of the local contributions from spin density on ^{13}C8. The analysis presented here gives a Mo···C distance whose value is expected to be ca. 2.7−2.9 Å in the “very rapid” intermediates formed with *both* xanthine and HMP, consistent with plausible bond lengths for a Mo−O−C8 fragment where C8 is a trigonal-planar aromatic carbon. The difference from earlier conclusions is explained. The data thus do not support the existence of a direct Mo−C bond in the signal-giving species. This conclusion supports a mechanism that does not involve such an interaction and which begins with base-assisted nucleophilic attack of the Mo^{VI}-OH group on the C-8 of substrate, with concomitant hydride transfer to the Mo═S group to give Mo^{IV}-SH; the EPR-active “very rapid” species then forms by one-electron oxidation and deprotonation to yield the EPR-detectable Mo^{V}OS(OR) species. We further discuss the complexities and limitations of the semiempirical method used to arrive at these conclusions.

Introduction

Xanthine oxidase is a molybdenum-containing enzyme involved in the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid.[1−3] The structural, functional, and mechanistic aspects of the protein have been extensively studied,[4−6] and the crystal structure of the enzyme from cow’s milk has been reported recently.[7] The active site of the enzyme can be formulated as LMo^{VI}OS(OH), where L is an enedithiolate ligand contributed by a pyranopterin cofactor that is common among all molybdenum enzymes other than nitrogenase, as well as all tungsten-containing enzymes. It has been known for some time that solvent water is the ultimate source of the oxygen atom incorporated into product,[8] but that the proximal oxygen donor in the catalytic sequence is a catalytically labile site on the enzyme that is regenerated in the course of each catalytic cycle with oxygen from solvent[9] and that corresponds to the oxygen of a Mo−OH group of the active site.[5]

A number of EPR signals attributable to the paramagnetic Mo(V) (d^1, $S = {}^1/_2$) state of the enzyme have been detected during catalysis,[1] and these have been investigated by EPR and related techniques such as ENDOR and ESEEM.[10−12] One of these, termed “very rapid” on the basis of the kinetics of its formation and decay during the reaction of enzyme with xanthine, has been shown to represent an authentic catalytic intermediate. It is formed to a varying degree with a number of substrates for the enzyme, including 2-hydroxy-6-methylpurine. With this slow substrate, the species giving rise to the “very rapid” EPR signal accumulates transiently to the extent of approximately 80% at pH 10, and it has been shown to represent an obligatory intermediate in the course of the reaction.[13]

[†] Northwestern University.

[‡] The Ohio State University.

[§] Present address: Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT.

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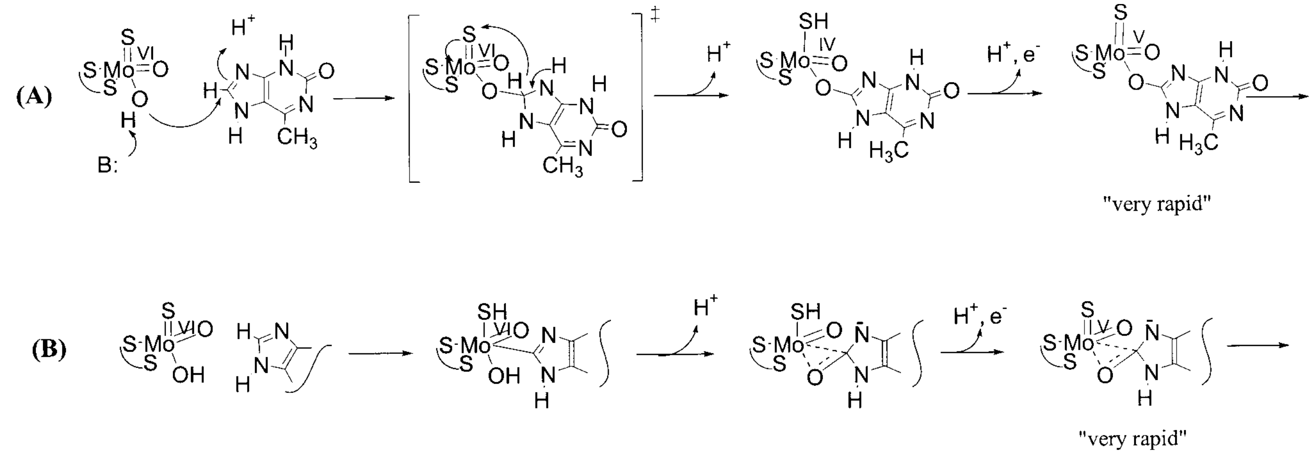
Scheme 1



Two alternate structures have been proposed for the species giving rise to the "very rapid" EPR signal, and these are incorporated into fundamentally different mechanisms. In the first mechanism, the product of hydroxylation (in the enolate tautomeric form) is proposed to be coordinated to the molybdenum via the newly introduced oxygen atom (Scheme 1A). The bracketed intermediate/transition state is proposed to form via base-assisted nucleophilic attack of the Mo^{VI}-OH group on the C-8 of substrate, with concomitant hydride transfer to the Mo═S group to give Mo^{IV}-SH.[5,14,15] The EPR-active "very rapid" species forms by one-electron oxidation and deprotonation to yield the EPR-detectable $Mo^{V}OS(OR)$ species. In the second mechanism (Scheme 1B), product is bound to the molybdenum as the keto tautomer in a side-on η_2 fashion.[11,16] The process leading to this structure begins with insertion of the C8−H bond across the Mo^{VI}═S bond to give a species with a Mo−C bond, a reaction with chemical precedent.[17] The "very rapid" intermediate is arrived at by subsequent insertion of oxygen across the Mo−C bond and (although not explicitly considered in the original paper) oxidation of the center by one equivalent.

A key distinction between these two mechanisms is whether there is a direct bonding interaction between Mo and C8 of the purine nucleus in the species giving rise to the "very rapid" EPR signal. ^{13}C isotope labeling provides a direct approach to this problem, and an elegant ^{13}C ENDOR study of the "very rapid" signal formed during the reaction of [$^{13}C8$] xanthine has been presented.[10,11] The reported analysis of the results gave a short Mo−C8 distance, which was interpreted as evidence for the existence of a Mo−C bond in this species.[11] The present report describes an independent examination of this issue. We describe in detail ^{13}C ENDOR measurements of the "very rapid" EPR signal generated by xanthine oxidase during reaction with 2-hydroxy-6-methylpurine ($^{13}C8$-HMP). The resulting $^{13}C8$ hyperfine tensor is discussed in the framework of a detailed consideration of factors involved in extracting metrical parameters from an anisotropic hyperfine interaction composed of contributions from multiple sources, in particular, the effect of local contributions from spin density on the ^{13}C nucleus. We demonstrate that the Mo−C8 distance is not unusually short in the "very rapid" species formed either with xanthine or HMP and explain the difference with the earlier analysis. As there is *no* Mo−C8 bond in the intermediate that gives rise to the "very rapid" EPR signal formed with either HMP or xanthine, there is no evidence that such a bond forms at any stage in the reaction of the enzyme.

Materials and Methods

Sample Preparation. Xanthine oxidase was purified using the method of Massey et al.[18] The paramagnetic "very rapid" intermediate was generated by reacting 2-hydroxy-6-methylpurine (HMP) with functional oxidase as described in Lorigan et al.[12] The 8-[^{13}C]-HMP was synthesized according to a procedure to be described elsewhere.[19]

EPR and ENDOR Spectroscopy. CW 35 GHz EPR and ENDOR measurements have been carried out on a modified Varian E-109 spectrometer in a dispersion mode with 100 kHz field modulation under "rapid passage" conditions.[20−22] Pulsed 35 GHz ENDOR spectra are recorded on a spectrometer described earlier.[23] Both spectrometers are equipped with a helium immersion dewar, and all the measurements were carried out approximately at 2 K. Pulsed ENDOR experiments employed the Davies ENDOR sequence ($\pi-T-\pi/2-\tau-\pi-\tau-$echo) where the radio frequency was applied during the interval T for a period t_{RF}.[24] The typical microwave pulse lengths used were $t = 80$ ns, $T = 132\ \mu s$, and $t_{RF} = 120\ \mu s$. The applied radio frequency was broadened in bandwidth by mixing with a 100 kHz noise to improve the signal-to-noise ratio.[25]

For a nucleus (N) of spin $I = {}^1/_2$ interacting with a paramagnetic center, the first-order ENDOR resonance condition for a single molecular orientation can be written as follows:

$$\nu_{\pm} = |\nu_N \pm A/2| \qquad (1)$$

where ν_N is the Larmor frequency and A is the orientation-dependent hyperfine coupling constant of the coupled nucleus. The equation predicts a doublet with separation A centered at ν_N when $\nu_N > A/2$, as is true here. The full hyperfine tensor of a coupled nucleus, both principal values and orientation parameters (Euler angles) with respect

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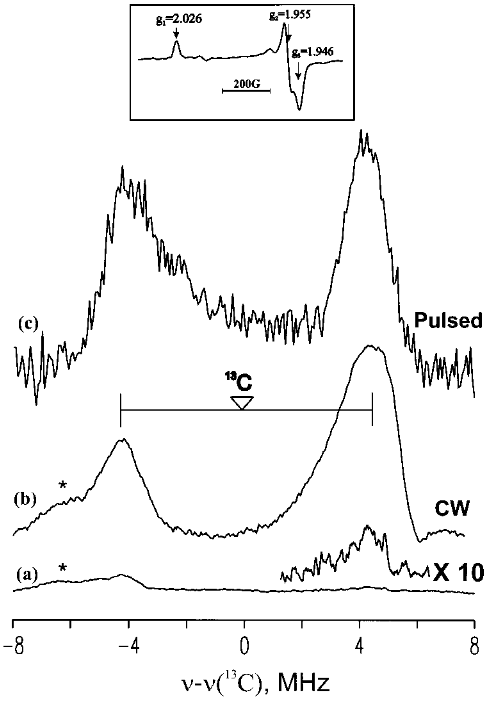

Figure 1. CW ENDOR spectra taken at the maximum EPR intensity of "very rapid" EPR signal generated with HMP: (a) natural-abundance HMP; (b) ^{13}C8-HMP. Conditions: $\nu_{\mathrm{MW}} = 35.165$ GHz, 2 G, 100 kHz field modulation. (c) Davies ENDOR of the ^{13}C-labeled sample at the maximum echo position. Conditions: $\nu_{\mathrm{MW}} = 34.754$ GHz; three-pulse microwave sequence ($t_{\mathrm{MW}}-T-t_{\mathrm{MW}}-\tau-t_{\mathrm{MW}}-\tau-$echo) with $t_{\mathrm{MW}} = 80$, 40, 80 ns, $T = 132$ μs, $\tau = 0.6$ μs and $t_{\mathrm{RF}} = 120$ μs during the interval, T. The applied radio frequency was broadened with 100 kHz bandwidth white noise.[25,46] (Inset) CW EPR spectrum of "very rapid" signal of xanthine oxidase generated with HMP, measurement was done at ~2 K under the dispersion-mode rapid-passage condition. Conditions: 2 G modulation, microwave frequency 35.165 GHz.

to the **g**-tensor frame, is obtained by simulating the 2-D pattern of orientation-dependent ENDOR spectra recorded across the EPR envelope using the procedures and program described elsewhere.[26−28] The computer simulation and analysis of the frozen-solution ENDOR spectra used in the present work are described in the same references.

Results

The 35 GHz CW EPR spectrum of the "very rapid" EPR signal of xanthine oxidase generated with HMP is given in Figure 1 (inset). The observed g-values are $g = [2.026, 1.955, 1.946]$ corresponding to an axially symmetric pattern with small rhombicity. These values are, within error, the same as those reported earlier.[29] The Q-band EPR spectrum of a sample labeled with ^{13}C at the C8 position of HMP was undistinguishable; hyperfine broadening has been seen in X-band spectra[19] and is comparable to that seen previously with 8-^{13}C-xanthine.[30]

35 GHz CW ENDOR spectra of natural abundance (unlabeled) and ^{13}C-labeled "very rapid" samples were recorded at the field of maximum EPR intensity, near $g_2 = 1.955$, and are given in Figure 1a,b respectively. The ENDOR spectrum of the ^{13}C-labeled sample (Figure 1b) consists of a doublet composed of ν_- and ν_+ branches centered around the ^{13}C Larmor frequency, $\nu_{\mathrm{C}} \sim 13$ MHz at this magnetic field. These signals are unequivocally from ^{13}C8 of HMP as they largely disappear in the unenriched sample (Figure 1a), although low-intensity ^{13}C-ENDOR signals in fact are seen with natural abundance ^{13}C-(1.1%).

In addition to the ^{13}C ENDOR signals, there is a broad signal seen with both samples near the $\nu_-(^{13}\mathrm{C})$ features, around −6.5 MHz (Figure 1a,b), whose counterpart is not seen in the ν_+ manifold. This signal is assigned to the double quantum transition ($\Delta m_{\mathrm{I}} = \pm 2$) of weakly coupled nitrogen, most likely from the substrate, HMP.[31] This ^{14}N feature is not observed in the pulsed ENDOR spectrum of ^{13}C-enriched sample because of the choice of pulse lengths; it is not discussed further. As is seen in Figure 1b, the $\nu_+(^{13}\mathrm{C})$ branch is more intense and better resolved than the $\nu_-(^{13}\mathrm{C})$ branch, and it does not overlap with the ^{14}N signal. For these reasons, the remainder of the analysis discusses only the ν_+ branch.

The ν_+ branch of the ENDOR features of the ^{13}C spectrum in Figure 1b is asymmetric with a tail toward the radio frequency sweep direction (high-to-low in this case) as is common.[31] This tailing arises from slow nuclear spin relaxation[22,31] and is typically accompanied by a shift of the center of the ENDOR pattern in the direction of the radio frequency sweep, the extent depending on the sweep rate. To correct for such an artifact, the frequencies reported here are averages from measurements carried out by sweeping the radio frequency in both the positive and negative directions.

A further difficulty presented by the sweep distortions is that determination of the ^{13}C8 hyperfine tensor requires the simulation of the peak shapes in a 2D field-frequency pattern of ENDOR spectra collected at multiple fields across the EPR envelope. To achieve conditions where the CW ENDOR spectra could be subjected to such an analysis properly, we compared them to Davies pulsed ^{13}C ENDOR spectra. The pulsed ENDOR technique exhibits substantially worse S/N than CW but does not suffer from the sweep artifacts. Thus, by comparing the pulse spectra with CW spectra collected under various conditions we were able to establish conditions under which reliable data could be collected in the CW mode. A representative Davies pulsed ^{13}C ENDOR spectrum recorded at $g_2 = 1.955$ is compared in Figure 1 with an optimized CW ^{13}C ENDOR spectrum taken at the same g-value with a negative sweep. While the ν_+ feature still exhibits a tailing to low frequency, comparison between the CW and pulsed spectra in this figure, as well as other data (not shown), shows that the high-frequency edge is not distorted by relaxation phenomena. Thus, we rely on the more readily acquired CW spectra for further analysis.

We performed orientation-selective CW ENDOR measurements to determine the complete hyperfine (**A**) tensor for ^{13}C8 of HMP and its orientation parameters (Euler angles) with respect to the **g**-tensor of the "very rapid" signal. Figure 2A represents CW ENDOR spectra of the ^{13}C-labeled sample recorded at selected magnetic fields across the entire EPR envelope. The top spectrum recorded at $g_1 = 2.026$ position is a "single-crystal-like" pattern associated only with those molecules for which the external field lies along g_1. Only one set of signals is observed, which shows that there is only one species (conformer) present. As the field is increased, a shoulder develops on the high-frequency edge of the ν_+ feature. The shoulder becomes more pronounced as the field is raised until about $g = 1.979$; upon further increase in the field the shoulder collapses and the ν_+ feature again becomes a single peak.

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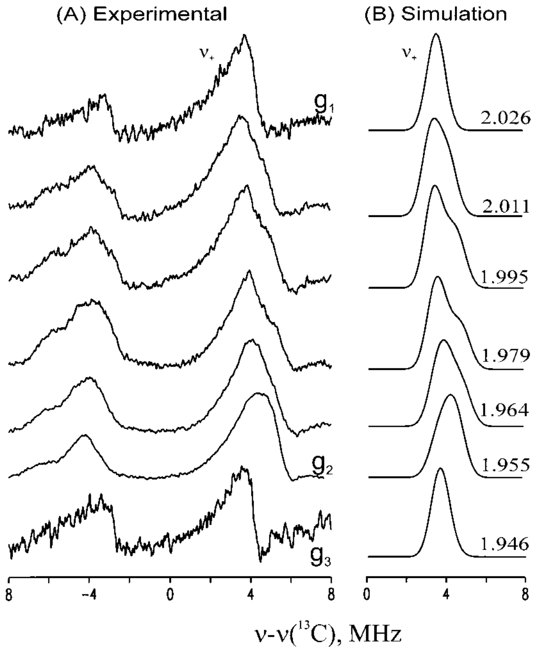

Figure 2. (A) The experimental orientation dependent ^{13}C ENDOR spectra of ^{13}C-labeled sample of HMP-generated “very rapid” signal recorded across the EPR envelope. Conditions: $\nu_{MW} = 35.165$ GHz, 2 G, 100 kHz field modulation. (B) Corresponding simulated ENDOR spectra. Only the ν_+ component of the simulation is given for clarity. The parameters used for the simulation are given in the text.

The pattern presented by this 2D field-frequency plot represents a hyperfine tensor that is mostly isotropic with an additional small anisotropic hyperfine coupling of more-or-less axial symmetry.[26] Accurate ^{13}C hyperfine tensor and orientation parameters were obtained by computer simulations of these spectra; the ν_+ component of the simulations is given in Figure 2B. The simulation gives principal values for the hyperfine tensor of $\mathbf{A} = [10.2(1), 7.0(1), 6.5(1)]$ MHz, with the unique values making an angle of $\beta = 65°$ with the g_1 direction. The hyperfine tensor of HMP corresponds to an isotropic coupling of $a_{iso} = 7.9(1)$ MHz, plus an anisotropic term that roughly exhibits the dipolar form, $\mathbf{T} = [2T, -T, -T]$, where $T = 1.15(25)$ MHz. It is noteworthy here that the hyperfine interaction tensor for ^{13}C8 of HMP is very similar to that reported for [8-^{13}C] of substrate xanthine in the corresponding state: the tensor for xanthine as substrate was reported to be axial, $\mathbf{A} = [11.1, 7.6, 7.6]$ MHz,[11] which gives $a_{iso} = 8.8$ MHz and $T = 1.17$ MHz.[32] The virtual identity of these ENDOR results confirms that the “very rapid” intermediates for these two substrates are equivalent in the vicinity of the Mo, and in the following discussion we treat them in parallel. In particular, for the remainder of this paper we treat the dipolar couplings for the two substrates as being identical, with $T = 1.15$ MHz, as any difference is far smaller than the joint uncertainties.

Discussion

The goal of the present effort is to characterize the species giving rise to the “very rapid” EPR signal, in particular, to help distinguish between two competing proposals for the catalytic mechanism by determining whether Mo and C8 are sufficiently close that there can be significant direct Mo−C8 η_2 (side-on) bonding interaction. We approach this problem in the context of our extensive use of ENDOR spectroscopy to study structures of enzymic intermediates and of bound substrates, products, and other small molecules.[33,34] In these prior studies, the hyperfine interactions of nuclei on enzyme-bound small molecules were dominated by their “nonlocal (nl)” electron−nuclear dipole interaction with the metal ion(s) in the enzyme active site, and in favorable cases we were able to extract precise metrical parameters for the small-molecule binding. *In principle*, the analysis of the anisotropic hyperfine interaction for the C8 of HMP and xanthine[11] can likewise give metrical information about the “very rapid” intermediate, because this interaction contains contributions which depend on metrical parameters of the intermediate, these structure-dependent terms taking the form of electron−nuclear dipolar interactions between ^{13}C8 and the unpaired electron spin localized in the Mo d_{xy} orbital (~65%), with a lesser contribution from the more remote and lesser spin on sulfur (~35%).[29] Unfortunately, these geometry-dependent contributions to the hyperfine interaction are not merely augmented, but comparable in size to a local (loc) contribution from the small spin density on C8 whose existence is established by the nonzero value for a_{iso}. As we now discuss, the semiempirical analysis necessary to decompose the hyperfine interaction does not permit, in the present case, the detail of our other studies. We illustrate the difficulties with such an effort through progressively more elaborate calculations of the Mo−C8 distance. Nonetheless, we show that the Mo−C8 distance in not anomalously short as thought earlier. We then discuss the mechanistic implications of this finding.

Analysis. The lowest level of approximation for treating the anisotropic hyperfine interaction at the ^{13}C8 nucleus of the purine ring would be to employ a point-dipolar model in which it is assumed that anisotropic contribution to the ^{13}C8 hyperfine tensor entirely arises from the nonlocal (nl) interaction between the unpaired spin density on Mo (ρ_{Mo}) and the ^{13}C8. This will generate an axial tensor in which the measured C8 hyperfine term, T, is determined by the Mo−^{13}C8 distance (r(Mo−C8)) through the following the equation:

$$T = \rho_{Mo} g_e \beta_e g_n \beta_n / r^3 = (0.65)20/r^3 \text{ MHz} \qquad (2)$$

where we follow Howes et al.,[11] who *implicitly* used the value $\rho_{Mo} = 0.65$.[35] The experimental value, $T = 1.15$ MHz, yields r(Mo−C8) = 2.24 Å for both HMP and xanthine.

As noted explicitly by Howes et al.,[11] the above treatment of T as being entirely nonlocal is not appropriate because one must first account for the local (loc) contribution from spin density on ^{13}C8, which not only gives rise to the isotropic coupling but also will produce an anisotropic one. This local anisotropic interaction, which has axial symmetry (see below), is characterized by an interaction constant (T_{loc}) which carries bonding, not metrical, information. One must characterize this interaction and use the result to extract from the measured tensor the residual through-space dipolar interaction constant, denoted T_{nl}. Only this latter is to be used in eq 2 (or its generalizations; see below) to estimate r(Mo−C8). Now, the dipolar interaction of a nucleus with the local spin on that atom will not in general be diagonal in the same coordinate frame that diagonalizes the nonlocal dipolar interaction with spin on an adjacent atom, and as a result the decomposition of an experimental tensor is not simple, in general.[36,37] However, the reported ^{13}C8 hyperfine interaction for xanthine is axial,[11] and the hyperfine tensor we measured

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for HMP (see above) is essentially axial. In this circumstance, the axial local and nonlocal dipolar interactions must have their unique directions in common (parallel) or nearly so.[38] Thus, to a first approximation we may merely subtract the local contribution from the observed anisotropic interaction constant to obtain the through-space term, $T_{nl} = T - T_{loc}$, which is then used in the determination of geometric parameters; we examine the validity of this approach presently.

We begin by noting that the spin density on C8 resides in an sp^n hybrid orbital on C8 whose coefficient in the half-filled HOMO (which exhibits mostly Mo(d_{xy}) character) is $(\rho_c)^{1/2}$, where ρ_c is the spin density on C8:

$$\Psi = (\rho_{Mo})^{1/2}\phi_{Mo} + ... + (\rho_c)^{1/2}\phi_c{}^n \tag{3}$$

with

$$\phi_c{}^n = (1/(1+n))^{1/2}(\phi_{2s} + n^{1/2}\,\phi_{2p})$$

In this description the isotropic and *local* anisotropic hyperfine coupling for ^{13}C8 can be written as

$$a_{iso} = \rho_c[1/(1+n)]a_o \quad T_{loc} = \rho_c[n/(1+n)]T_o \tag{4}$$

where $a_o = 3777$ MHz is the isotropic hyperfine coupling for one electron in the 2s orbital on C and $T_o = 107$ MHz is the anisotropic hyperfine couplings for one electron in a 2p orbital on C.[39] The C8 carbon of the species giving rise to the "very rapid" EPR signal as pictured in Scheme 1 is trigonal, and the experimental tensor shows that the spin density must be introduced through σ delocalized into the in-plane orbital whose hybridization is nominally $n = 2$. However, the hybridization constant, n, could be larger than 2, perhaps approaching $n = 3$, if such bonding were strong. If T_{loc} were known, one could solve eq 4 for ρ_c and the apparent hybridization, n, but in fact we measure only a sum of the local and the nonlocal, dipolar, terms, $T = T_{loc} + T_{nl}$, and wish to decompose this sum. Thus, we retain n as a parameter, calculating ρ_c from the measured a_{iso} as a function of n, through use of eq 4. As we shall see, this hybrid spin-bearing orbital on C8 accurately reproduces the *form* of the experimental hyperfine interaction tensor: axial or approximately so, and with the isotropic interaction dominant ($a_{iso}/T_{loc} \geq a_{iso}/T$ = [6.9, HMP; 7.7 xanthine]. We note that there cannot be any significant π delocalization, because spin density in a 2pπ orbital of carbon gives a hyperfine interaction in which the isotropic coupling does not dominate, but rather one with a much smaller ratio of isotropic to anisotropic contributions, $a_{iso}/T_{loc} \sim 1.5$.[39-41]

The values of ρ_c for the "very rapid" intermediate of xanthine oxidase formed with HMP are presented in Table 1 not only for the nominal hybridization, $n = 2$, but also for $n = 1,3$, along with the decomposition of $T(^{13}C8) = 1.15$ MHz into local and nonlocal contributions. The r(Mo−C8) distance, also listed in Table 1, was calculated from the nonlocal term through use of eq 2. One first sees that, regardless of the value of n, a large percentage of the measured $T(^{13}C8) = 1.15$ MHz comes from the local contribution of the exceedingly small spin density on ^{13}C8. The distances calculated in this way from the residual T_{nl} for the nominal hybridization, $n = 2$, which are a much better approximation than that obtained when ignoring the local spin, are r(Mo−C8) ≳ 2.7 Å for both substrates. This is *significantly* longer than r(Mo−C8) < 2.4 Å reported originally[11] for xanthine, whose value can be seen from Table 1 to have been obtained because of the unphysical choice of $n = 1$.[35]

Table 1. Decomposition of the Anisotropic Interaction Parameter, $T(^{13}C8) = 1.15$ MHz, for the "Very Rapid" Intermediate of Xanthine Oxidase, As Formed with HMP and Xanthine,[11] into Local (loc) and Nonlocal, Dipolar (nl) Contributions, along with the Resulting Spin Density on C8 (ρ) and Mo−C8 Distance, Calculated through eqs 4 and 2, as a Function of the Hybridization, n (see text)[a]

	ρ (%)		T_{loc} (MHz)		T_{nl} (MHz)		r(Mo−C8) (Å)	
n	HMP	Xan	HMP	Xan	HMP	Xan	HMP	Xan
1	0.42	0.47	0.22	0.25	0.93	0.92	2.41	2.42
2	0.63	0.70	0.45	0.49	0.70	0.66	2.65(15)[b]	2.71
3	0.85	0.93	0.67	0.74	0.48	0.41	3.00(20)[b]	3.17

[a] Note, the decompositions and resulting r(Mo−C8) differ slightly for HMP and xanthine because of their different isotropic coupling constants, $a(^{13}C8) = 7.9$ MHz for HMP, but 8.8 MHz for xanthine.[11] [b] The uncertainties in r(Mo−C8) for HMP apply *within the semiempirical model* discussed here, using uncertainties in the T_{nl} equal to those ascribed to $T(^{13}C8) \pm 0.1$ MHz. No uncertainties were reported for the hyperfine tensor for xanthine; our experience suggests they cannot be appreciably less than those for HMP.

As an important aside, given this change in r(Mo−C8), we also reexamined the Mo−C distance reported by Howes et al.[11] for the "formaldehyde-inhibited" XO intermediate, which is formed during reaction with formaldehyde and which presumably corresponds to the "very rapid" intermediate. Taking the experimental ^{13}C hyperfine tensor and $n = 1$[11] gives the reported dramatically short value of r(Mo−C) = 1.95 Å; however, taking the more reasonable hybridization value, $n = 2$, gives a more reasonable distance, r(Mo−C) = 2.4 Å. Note, moreover, because the ^{13}C coupling is so large, the implied distance is extremely sensitive to the assumed hybridization ($n = 3$; r(Mo−C) > 4 Å) and detailed analysis in this case is not particularly reliable. Thus, we suggest that the results for this intermediate cannot be used to infer the presence of a strong Mo−C interaction. We further note in passing a curious internal "inconsistency" of this analysis as it relates to the proposal of direct Mo−C bonding. As the Mo−C interaction increases, one might well expect the geometry at C to become increasingly nonplanar. To the extent that the bonding is described by this model, the stronger bond would thus result in an increase in hybridization constant n, but in the context of this analysis this would lead to a longer calculated value for r(Mo−C8), Table 1, and thus an apparently weaker interaction.

This semiempirical analysis is not complete, but it will be seen that we have introduced the major corrections to the dipolar interaction and thus determined the essence of the final answer. Thus, it is reasonable to ask, does the analysis yield a plausible geometry for a Mo−O−C8 fragment with a trigonal-planar C8 carbon and without significant metal−carbon interactions? In fact, use of a C8−O distance of 1.4 Å, an Mo−O distance of

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(36) We have published an exact treatment that embodies the proper approach for describing the hyperfine tensor for a nucleus that experiences two noncollinear dipolar interactions.

(37) Willems, J.-P.; Lee, H.-I.; Burdi, D.; Doan, P. E.; Stubbe, J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1997**, *119*, 9816−9824.

(38) A limiting condition, in which two dipolar interactions with orthogonal unique directions have equal dipolar interaction constants, accidentally gives rise to a dipolar tensor whose unique axis is orthogonal to those of the contributing tensors, but it is easy to show that this does not occur here.

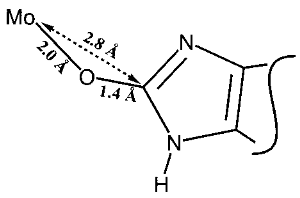
(39) Weil, J. A.; Bolton, J. R.; Wertz, J. E. *Electron Paramagnetic Resonance: Elementary Theory and Practical Applications*; John Wiley & Sons: New York, 1994.

(40) Carrington, A.; McLachlan, A. D. *Introduction to magnetic resonance with applications to chemistry and chemical physics*; Harper & Row: New York, 1967.

(41) One might also expect more π-delocalization from an η-2 interaction with Mo, as well.

Chart 1

Idealized Mo-O-C Fragment



~2 Å, and a tetrahedral angle at O of 109° defines an Mo−O−C8 linkage with r(Mo−C) = 2.8 Å, as depicted in Chart 1.[35,29]

Proceeding, however, with the correction process, one must treat the effects of spin density on the bridging oxygen atom, ρ_O. With the spin density on C8 approaching 1% (Table 1), one would expect no less than a few percent on the bridging oxygen which is bound directly to the d^1 Mo ion and the short O−C bond length of $d \sim 1.4$ Å would give this spin a disproportionately large weight. In fact, ^{17}O ENDOR measurements on a labeled "very rapid" intermediate[11] show that ^{17}O in this position exhibits an isotropic coupling, $a_{\mathrm{iso}} = 38$ MHz. This value with the appropriate reference hyperfine coupling for one electron in a 2s orbital on O, assuming the spin is in the orbital(s) with $n = 3$[42] and using the left side of eq 4, gives $\rho_O \sim 3\%$. Taking a point dipole approximation for the interaction of spin on O with C8 gives $T_O \sim 7.3\rho_O$ MHz,[43,44] with the result that $T_O \sim 0.2$ MHz. Subtracting this from the T_{nl} in the table would lengthen the distance calculated for $n = 2$ to r(Mo−C8) ~ 2.9−3.0 Å and would lengthen the distance for $n = 3$ even more.

Next, we note that in such a Mo−O−C8 fragment as illustrated in Chart 1, the unique axis of the local dipolar interaction would lie along the C8−O bond, while that of the nonlocal interaction with spin on Mo would lie along the Mo−C8 vector. These two make an angle of <~40°, which would break the axial symmetry of the net hyperfine interaction tensor. However, exact diagonalization of the two tensors, done by modifying in an obvious way our exact formulas for the dipolar tensor of a nucleus interacting with the two electron spins of a diiron center,[37] shows that the tensor, though rhombic, still has a distinctly axial character and that the rhombicity introduced is compatible with experimental results (see above).The additional effect of this nonaxiality would be to lessen the resulting principal value, such that the proper decomposition would lead to a smaller value of r(Mo−C8), by as much as 0.2 Å. However, this effect is opposed by the correction form the dipolar interaction with the spin density on the sulfur atom (~35%).[29] Again using the modified diagonalization procedure, we find that this interaction would lead to "lengthening" of the inferred Mo−C8 distance; the amount would be $\lesssim 0.1$ Å, varying somewhat with the C8−Mo−S angle which likely is somewhat greater than ~90°. The net of the three additional corrections, spin on O, on S, and noncollinearity in tensor decompositions, thus is likely to be perhaps an ~0.1 Å increase in the Mo−C8 distances in Table 1 for $n = 2$, giving a "best estimate" value of r(Mo−C8) ~2.8 Å for this hybridization and a larger value for larger n.

Overall, the semiempirical approach thus shows that the ENDOR data for the "very rapid" intermediate is fully compatible with the structure of Chart 1, and an analogous conclusion holds for the "formaldehyde-inhibited" state, as well. Could, or even should, this rather satisfying picture be refined further, for example, by self-consistently treating all the correction steps, which was *not* done above? Our answer: No, the "theory" itself is not that robust. The assumption that the observed isotropic and anisotropic interactions can be related according to eq 4 to reference values for a single electron in a 2s and a 2p orbital are oversimplifications. In particular, as we showed long ago in the case of the ^{14}N hyperfine coupling in M(TPP), M = Ag, Cu,[45] the polarization of the inner shell substantially modifies the observed isotropic coupling. A more elaborate treatment yielded a value for the spin density in an sp^n orbital on nitrogen that is *not* accurately related to the observed isotropic coupling through eq 4. To model the "better" spin density with eq 4 would require use of an "effective" value of a_o that is roughly $^3/_4$ that used above, an effect which is equivalent to an increase in hybridization, n and which increases r(Mo−C). Any effort at increased rigor would require the more complicated approach we used previously. But in that early case we were working with ^{14}N hyperfine coupling where the structure of the Cu(TPP) was well-known, the local interaction was overwhelmingly dominant, and we were interested in inferences about bonding, not metrical parameters. Such a treatment is simply not possible for the "very rapid" intermediate, where no single contribution dominates the small ^{13}C8 coupling.

Conclusions. The analysis presented here gives a Mo···C distance whose value is expected to be ca. 2.7−2.9 Å in the intermediates formed with *both* xanthine and HMP, consistent with plausible bond lengths for a Mo−O−C8 fragment where C8 is a trigonal-planar aromatic carbon (Chart 1). The data thus do not support the existence of a direct Mo−C bond in the signal-giving species, and by inference in the intermediate that precedes it in the catalytic sequence, and therefore do support the mechanism of Scheme 1A over that of Scheme 1B. We have further discussed the complexities and limitations of the semiempirical method used to arrive at these conclusions.

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(42) To use the experimental anisotropic hyperfine coupling to deduce the hybridization on O would involve the same issues of decomposition as faced for C8. As the correction from spin on O is minor, we use this reasonable assumption; alternate assumptions would not change our conclusions.

(43) Calculations with the formulas for distributed spin in the 2s and 2p σ orbitals (ref) have been carried out and reduce this value only modestly, with the change depends on the details of the hybridization on O. Hence we ignore this "pseudo-refinement".

(44) McConnell, H. M.; Strathdee, J. *Mol. Phys.* **1959**, *2*, 129−138. For Erratum, see: Pitzer, R. M.; Kern, C. W.; Lipscomb, W. N. *J. Chem. Phys.* **1962**, *137*, 267.

(45) Brown, T. G.; Hoffman, B. M. *Mol. Phys.* **1980**, *39*, 1073−1109.

(46) Doan, P. E. Manuscript in preparation.