^{13}C-ENDOR Studies of the Inhibited Species of Xanthine Oxidase: The First Direct Evidence for a Molybdenum-Carbon Bond in a Biological System

Barry D. Howes,[1ac] Brian Bennett,[1ad] Robert C. Bray,[1a] Raymond L. Richards,[1b] and David J. Lowe*,[1b]

School of Chemistry and Molecular Sciences University of Sussex, Brighton, BN1 9QJ, U.K. Nitrogen Fixation Laboratory University of Sussex, Brighton, BN1 9RQ, U.K.

Received June 13, 1994

Xanthine oxidase[2] is the most extensively studied of molybdenum enzymes that depend on the pterin molybdenum cofactor.[3] No three-dimensional structure is yet available, but progress has recently been reported[4] for a related aldehyde oxidoreductase. Much information is nevertheless available about the enzyme structure in the immediate vicinity of molybdenum and about the enzymic reaction mechanism, from spectroscopic methods, particularly EPR and EXAFS (extended X-ray absorption fine structure). In oxidized xanthine oxidase molybdenum is in the Mo(VI) oxidation state, bearing an oxo and a sulfido ligand. Reaction with substrates reduces the metal to the Mo(IV) and Mo(V) states. Several clearly defined and structurally distinct Mo(V) species from the reduced enzyme have been distinguished by EPR spectroscopy. Substitution with stable isotopes has facilitated attempts to deduce the structures of these species as well as investigations of their kinetic relationships and hydrogen- and oxygen-transfer reactions.[2b] Molybdenum species so investigated include one, named "Very Rapid", that represents a transient intermediate in the catalytic reaction of xanthine or certain other substrates with the molybdenum center. Another species, named "Inhibited", represents the product of an inhibitory side reaction of formaldehyde or other aldehyde substrates with this center. Understanding[2b,5] of the catalytic mechanism depends in substantial measure on these studies, supplemented by studies of model compounds and model reactions.[6]

ENDOR (electron-nuclear double resonance) spectroscopy[7] complements EPR in the analysis of ligand hyperfine couplings, being particularly valuable in the case of weak couplings. We recently extended investigations of Mo(V) forms of xanthine oxidase to include ENDOR studies.[8] We now describe ENDOR analyses of Mo(V) species from the enzyme, prepared with the use of ^{13}C-labeled aldehydes and aiming to provide a more complete knowledge of the local structure around molybdenum. The data presented indicate Mo-C bond formation, the first direct evidence for such a bond in a biological system. A revised structure for the Inhibited signal-giving species is proposed, and the relevance of the results to the reaction mechanism of the enzyme is discussed.

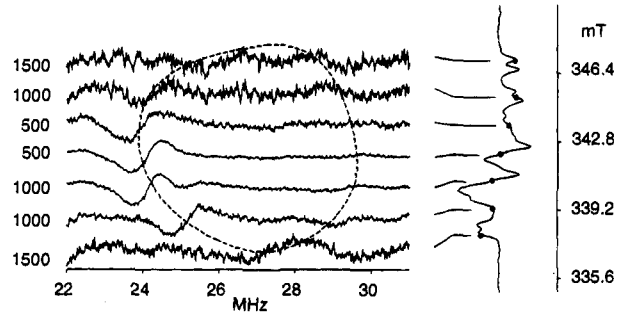

Figure 1. ENDOR spectra from the Inhibited signal from treatment of xanthine oxidase with H^{13}CHO, recorded at field settings, as indicated on the EPR spectrum plotted on the right, with the magnetic field scale indicated vertically. The dashed line indicates approximately the movement of the extreme features of the ^{13}C ENDOR pattern as the field is varied. The number of scans done for each trace is indicated on the left. Traces were normalized to the same overall amplitude. Recording conditions were as follows: temperature, 25 K; microwave frequency, 9.46 GHz; microwave power, 5 mW; rf modulation depth, 100 kHz; rf attenuation, 0 dB; scan rate, 0.43 MHz s^{-1}.

A series of ^{13}C ENDOR spectra[9] were recorded at intervals across the whole EPR envelope of the xanthine oxidase Inhibited signal[10] generated with ^{13}C-labeled formaldehyde. The high-frequency halves are shown in Figure 1. Visual inspection of the spectra enables the ^{13}C hyperfine coupling tensor to be determined[11] as $A_{1,2,3}$, 52.5, 40.6, 40.6 MHz; A_{iso}, 44.5 MHz.[12] These values are more reliable than those determined earlier[10e] by EPR for the identical Inhibited signal, developed with [^{13}C]-methanol. Analysis of this hyperfine tensor using the point dipole approximation provides an estimate of 1.7 Å for the Mo-C distance, r. The breakdown[7a] of this approximation, due to electron delocalization and for distances of the order of 2 Å, can be allowed for, respectively, by including the effects of

(1) (a) Chemistry and Molecular Sciences. (b) Nitrogen Fixation Laboratory. (c) Present address: Laboratorio di Spettroscopia Molecolare, Dipartimento di Chimica, Universita Degli Studi di Firenze, Italy. (d) Present address: School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K.

(2) (a) Bray, R. C. *Enzymes (3rd Ed.)* **1975**, *12*, 299-420. (b) Bray, R. C. *Q. Rev. Biophys.* **1988**, *21*, 299-329. (c) Hille, R.; Massey, V. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; Wiley: New York, 1985; pp 443-518. (d) Hille, R. *Biochim. Biophys. Acta* **1994**, *1184*, 143-169. (e) Wootton, J. C.; Nicolson, R. E.; Cock, J. M.; Walters, D. E.; Burke, J. F.; Doyle, W. A.; Bray, R. C. *Biochim. Biophys. Acta* **1991**, *1057*, 157-185.

(3) Rajagopalan, K. V. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1991**, *64*, 215-290.

(4) (a) Ramao, M. J.; Barata, B. A. S.; Andrew, M.; Cobeck, K.; Moura, I.; Carrondo, M. A.; LeGall, J.; Lottspeich, F.; Haber, R.; Moura, J. J. G. *Eur. J. Biochem.* **1993**, *215*, 729-732. (b) Thoenes, U.; Flores, O. L.; Neves, A.; Devreese, B.; Van Beemen, J. J.; Huber, R.; Ramao, M. J.; LeGall, J.; Moura, J. J. G.; Rodriguez-Pousada, C. *Eur. J. Biochem.* **1994**, *220*, 910-911.

(5) (a) Turner, N. A.; Bray, R. C.; Diakun G. P. *Biochem. J.* **1989**, *260*, 563-571. (b) Pilato, R. S.; Stiefel, E. I. In *Bioinorganic Catalysis*; Reedijk, J., Ed.; Dekker: New York, 1993; pp 131-188.

(6) (a) Holm, R. H. *Coord. Chem. Rev.* **1990**, *100*, 183-221. (b) Greenwood, R. J.; Wilson, G. L.; Pilbrow, J. R.; Wedd, A. G. *J. Am. Chem. Soc.* **1993**, *115*, 5385-5392. (c) Enemark, J. H.; Young, C. G. *Adv. Inorg. Chem.* **1993**, *40*, 1-88. (d) Coucouvanis, D.; Toupadakis, A.; Lane, J. D.; Koo, S. M.; Kim, C. G.; Hadjikyriacou, A. *J. Am. Chem. Soc.* **1991**, *113*, 5271-5282.

(7) (a) Schweiger, A. *Struct. Bonding* **1982**, *51*, 1-119. (b) Lowe, D. J. *Prog. Biophys. Mol. Biol.* **1992**, *57*, 1-22.

(8) (a) Howes, B. D.; Pinhal, N. M.; Turner, N. A.; Bray, R. C.; Anger, G.; Ehrenberg, A.; Raynor, J. B.; Lowe, D. J. *Biochemistry* **1990**, *29*, 6120-6127. (b) Howes, B. D.; Bennett, B.; Koppenhöfer, A.; Lowe, D. J.; Bray, R. C. *Biochemistry* **1991**, *30*, 3969-3975. (c) Howes, B. D.; Bennett, B.; Lowe, D. J.; Bray, R. C. In *Flavins and Flavoproteins*; Curti, B., Ronchi, S., Zanetti, G., Eds.; De Gruyter: Berlin, 1991; pp 691-694.

(9) ENDOR spectra were recorded as described in 8b previously.

(10) (a) Xanthine oxidase, prepared from bovine milk by denaturation with sodium salicylate[10b], was ≈70% functional, and reaction with formaldehyde to give the Inhibited signal was essentially as described previously.[8b] The final concentration of ≈0.8 mM Mo(V) corresponded to ≈50% conversion of the functional enzyme to the signal-giving species. Examination of the EPR line shape (Figure 1) and comparison with earlier work[10c-f] confirms that a single chemical species is responsible for the ^{1}H- and ^{13}C-split rhombic signal. [^{13}C]Paraformaldehyde [(H^{13}CHO)$_n$, isotopic purity >98%, from MSD Isotopes] was heated in H_2O in a sealed tube at ≈120 °C for 4 h. (b) Hart, L. I.; McGartoll, M. A.; Chapman, H. R.; Bray, R. C. *Biochem. J.* **1970**, *116*, 851-864. (c) Pick, F. M.; McGartoll, M. A.; Bray, R. C. *Eur. J. Biochem.* **1971**, *18*, 65-72. (d) Morpeth, F. F.; Bray, R. C; *Biochemistry* **1984**, *23*, 1332-1338. (e) Tanner, S. J.; Bray, R. C.; Bergmann, F. *Biochem. Soc. Trans.* **1978**, *60*, 1328-1330. (f) Bray, R. C.; Gutteridge, S. *Biochemistry* **1982**, *21*, 5992-5999.

(11) (a) Hoffman, B. M.; Martinsen, J.; Venters, R. A. *J. Magn. Reson.* **1984**, *59*, 110-123. (b) True, A. E.; Nelson, M. J.; Venters, R. A.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1988**, *110*, 1935-1943.

(12) Simulated ENDOR spectra are consistent with estimated errors of ±0.2 MHz; Euler angles relating the hyperfine tensor to the **g** tensor have not been determined, but clearly from the experimental behavior, one or more is non-zero (cf. ref 11).

0002-7863/94/1516-11624$04.50/0

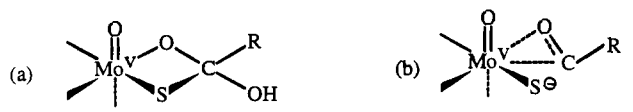

Figure 2. Structures proposed for the Inhibited Mo(V) EPR signal-giving species: (a) from earlier work[8a] and (b) from the present work.

electron delocalization on the carbon p-orbitals and by calculating the point dipole interaction using explicitly the molybdenum ground state d_{xy} orbital for the unpaired electron. In the present case not enough is known about the local structure for such a full calculation to be feasible, although a decreased spin density at Mo would decrease the estimated value of r. The effects of electron density on the carbon p-orbitals are amenable to calculation;[13,14] nevertheless, the lack of a detailed local structure means that a number of approximations must be made, so that the corrected maximum Mo−C distance of approximately 1.9 Å still has a degree of uncertainty. An alternative approach is to consider the feasibility of the previously proposed[5a,8a] structure for the Inhibited species, with a −Mo−O−C−S− ring arrangement (Figure 2a). The minimum Mo−C distance consistent with such a structure is approximately 2.5 Å,[16a] which, from a point dipole calculation, would have an associated hyperfine anisotropy of approximately 3.7 MHz. The measured anisotropy is 11.9 MHz, requiring a correction of 8.1 MHz to make a ring structure feasible. Such a correction is very unlikely to result from the anisotropic hyperfine interaction of electron density on the carbon p-orbitals for such a structure at this distance, especially when the maximum dipolar hyperfine component is estimated to be less than 2 MHz. The data are thus consistent with a short Mo−C distance (≈1.9 Å) and quite inconsistent with a larger one (≈2.5 Å), as in a ring structure.[16b]

Results similar to those obtained with formaldehyde have been obtained for the Inhibited signal arising from acetaldehyde[17] ($CH_3{}^{13}CHO$, data not shown).

^{13}C ENDOR thus provides strong evidence in favor of a direct Mo−C bond[18] in the Inhibited species. Speculations on such bonds in xanthine oxidase species were dismissed[5a,19] for lack of positive evidence. Figure 2b shows the structure now considered most likely for the Inhibited species; the carbonyl group is side-on to the molybdenum, and the aldehydic proton has been eliminated. A close analogy for such a structure, at the Mo(IV) level, and for the reaction giving rise to it, is provided by the work of Müller *et al.*[20] These workers determined the structure of the compound $[(C_6H_5)_4P]_2[Mo(NO)(OCN(CH_3)_2)(NCS)_4]$ in which the Mo−C bond length is 2.029 Å, a little longer than our estimate of that in the enzyme species. Significantly, in this compound, a carbonyl group is bonded side-on to molybdenum[21] and is[22] in the form of a carbamido moiety, derived by hydrogen elimination from dimethylformamide. In this context, it is particularly interesting that formamide and formate are both known substrates of xanthine oxidase.[23]

EPR and EXAFS provide further support of the structure of Figure 2b for the Inhibited species. EXAFS indicates[5a] an oxo ligand at molybdenum, while EPR with ^{17}O-substitution[10f] showed the presence of two weakly coupled oxygen atoms.[24] When the Inhibited signal is developed with formaldehyde (Figure 2b, R = H), one formaldehyde proton is strongly coupled to molybdenum. This proton does not exchange with the solvent and is not detected when the signal is developed with acetaldehyde or other aldehydes.[17] The present work provides no support for the carbon-bound hydroxyl group postulated[8a] as replacing the aldehydic proton (Figure 2a). Thus, ENDOR of the formaldehyde Inhibited species developed in ^2H_2O was not different from that prepared in ^1H_2O (data not shown).

The Inhibited species is relatively stable and is not an intermediate in the turnover of xanthine oxidase but the product of an inhibitory side reaction. Of more direct relevance to the catalytic cycle is the structure of the catalytically competent Very Rapid species.[2b] This was also assumed[6b,19b,25,26] to have a Mo−O−C structure, where the C is the 8-C of the substrate xanthine. However, in the light of the present work, a Mo−C structure must clearly be considered. If this could be established by further ENDOR work,[27] it would have important implications for the catalytic mechanism. In particular, postulated precursors of the Very Rapid species would arise by formal addition of the elements of the substrate molecule, RH, across the Mo=S double bond, to give a Mo(VI) species.[28] This would occur[6d] by concerted electrophilic attack by molybdenum on the 8-C carbon and proton abstraction by the sulfido group. Thus, Very Rapid precursors would have the metal in the Mo(VI) rather than the Mo(IV) state as has hitherto been assumed.[2b,29]

Acknowledgment. Support for a Linked Research Group was from the Agricultural and Food Research Council. R.C.B. thanks the Leverhulme Trust for an Emeritus Fellowship. We thank Dr. M. R. Hyman, Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, OR, whose unpublished work we quote.

(13) Snetsinger, P. A.; Chasteen, N. D.; van Willigen, H. *J. Am. Chem. Soc.* **1990**, *112*, 8155−8160.

(14) In addition to the dipolar superhyperfine interaction between molybdenum and carbon there are a number of other, generally small, interactions that may contribute to measured anisotropic coupling (A_{aniso}). These are due to electron density on the ^{13}C p-orbitals resulting from σ and π bonding. The measured isotropic coupling may be used to estimate, from tabulated values,[15] the electron percentage on the carbon 2s orbital. Subsequently, for an assumed carbon hybrid molecular orbital, one can estimate the electron percentage on the carbon p-orbitals and use these values to calculate A_σ and A_π, the hyperfine interactions on the carbon due to σ and π bonding, respectively. The effects of electron density on the carbon atom can now be removed from the measured A_{aniso} and the Mo−^{13}C separation redetermined.

(15) Morton, J. R.; Preston, K. F. *J. Magn. Reson.* **1978**, *30*, 577−582.

(16) (a) In the complex $[MoO(S_2CSPr^i)_2]$ there is a related ring system, −Mo−S−C−S−, in which the Mo−C distance (involving a weak, essentially π Mo−C interaction) is 2.25(1) Å: Hyde, J.; Venkatasubramanian, K.; Zubieta, J. *Inorg. Chem.* **1978**, *17*, 414−426. (b) Mo−C 2.25 Å[16a] requires the improbably high correction of 6.8 MHz to the measured hyperfine anisotropy.

(17) Malthouse, J. P. G.; Williams, J. W.; Bray, R. C. *Biochem. J.* **1981**, *197*, 421−425.

(18) (a) Mo−C bond lengths, in complexes generally not relevant to the enzyme, vary from about 1.82 Å (Mo−C multiple bonds)[18b] to 2.29 Å.[18c] (b) Lemos, M. A. N. D. A.; Pombeiro, A. J. L.; Hughes, D. L.; Richards, R. L. *J. Organomet. Chem.* **1992**, *434*, C6−C9. (c) Cotton, F. A.; Troup, J. M.; Webb, T. R.; Williamson, D. H.; Wilkinson, G. *J. Am. Chem. Soc.* **1974**, *96*, 3824−3828.

(19) (a) Bray, R. C. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum: New York, 1980; Vol. 2, pp 45−84. (b) George, G. N.; Bray, R. C. *Biochemistry* **1988**, *27*, 3603−3609.

(20) Müller, A.; Seger, U.; Werner, E. *Inorg. Chim. Acta* **1979**, *32*, L65−L66.

(21) (a) Side-on Mo−(η^2-COR) structures are well-established[21b,c] among complexes of Mo in the lower oxidation states. (b) Gambarotta, S.; Floriani, C.; Chiesi-Villa, A.; Guastini, C. *J. Am. Chem. Soc.* **1985**, *107*, 2985−2986. (c) Carmona, E.; Sanchez, L.; Martin, J. M.; Povenda, M. L.; Atwood, J. L.; Priestar, R. D.; Rogers, R. D. *J. Am. Chem. Soc.* **1984**, *106*, 3214−3222.

(22) (a) Side-on coordination of alkenes and alkynes to Mo is well known. Relevance of this to xanthine oxidase action is indicated by the finding that acetylene is an inhibitor of the enzyme (M. R. Hyman, unpublished work). The structure of an oxo−thiolato−Mo(V) model compound $[MoO(SC_6H_2Pr^i_3)_2(\eta^2\text{-}HCCC_6H_4CH_3\text{-}4)\{\eta^1\text{-}C(C_6H_4CH_3\text{-}4)CH(PMePh_2)\}]$ has recently been determined.[22b] Mo−C distances are in the range 2.02−2.21 Å. (b) Hughes, D. L.; Marjani, K.; Richards, R. L. *Abstracts of Papers, 16th International Conference on Organometallic Chemistry, University of Sussex, U.K.* Royal Society of Chemistry: Cambridge, 1994; p 247.

(23) (a) Morpeth, F. F.; George, G. N.; Bray, R. C. *Biochem. J.* **1984**, *220*, 235−242. (b) Anderson, R. F.; Hille, R.; Massey, V. *J. Biol. Chem.* **1986**, *261*, 15870−15876.

(24) The published ^{17}O hyperfine coupling data are not adequate to furnish reliable distance estimates but are consistent with Figure 2b.

(25) Gutteridge, S.; Bray, R. C. *Biochem. J.* **1980**, *189*, 615−623.

(26) Hille, R. *ACS Symp. Ser.* **1993**, *535*, 22−37.

(27) Preliminary ^{13}C ENDOR results on the Very Rapid signal were interpreted[8c] as being more consistent with a Mo−O−C, than with a Mo−C structure. However, these studies were not definitive, and further work, including ENDOR with ^{17}O, is in progress.

(28) Such a step was suggested, but not favored previously.[5a]

(29) McWhirter, R. B.; Hille, R. C. *J. Biol. Chem.* **1991**, *266*, 23724−23731.