Evidence Favoring Molybdenum−Carbon Bond Formation in Xanthine Oxidase Action: ^{17}O- and ^{13}C-ENDOR and Kinetic Studies†

Barry D. Howes,‡,§ Robert C. Bray,*,‡ Raymond L. Richards,|| Nigel A. Turner,‡ Brian Bennett,‡ and David J. Lowe*,||

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

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ABSTRACT: The reaction mechanism of the molybdoenzyme xanthine oxidase has been further investigated by ^{13}C and ^{17}O ENDOR of molybdenum(V) species and by kinetic studies of exchange of oxygen isotopes. Three EPR signal-giving species were studied: (i) Very Rapid, a transient intermediate in substrate turnover, (ii) Inhibited, the product of an inhibitory side reaction with aldehyde substrates, and (iii) Alloxanthine, a species formed by reaction of reduced enzyme with the inhibitor, alloxanthine. The Very Rapid signal was developed either with [8-^{13}C]xanthine or with 2-oxo-6-methylpurine using enzyme equilibrated with [^{17}O]H_2O. The Inhibited signal was developed with $^2H^{13}C^2HO$ and the Alloxanthine signal by using [^{17}O]H_2O. Estimates of Mo−C distances were made, from the anisotropic components of the ^{13}C-couplings, by corrected dipolar coupling calculations and by back-calculation from assumed possible structures. Estimated distances in the Inhibited and Very Rapid species were about 1.9 and less than 2.4 Å, respectively. A Mo−C bond in the Inhibited species is very strongly suggested, presumably associated with side-on bonding to molybdenum of the carbonyl of the aldehyde substrate. For the Very Rapid species, a Mo−C bond is highly likely. Coupling from a strongly coupled ^{17}O, not in the form of an oxo group, and no coupling from other oxygens was detected in the Very Rapid species. No coupled oxygens were detected in the Alloxanthine species. That the coupled oxygen of the Very Rapid species is the one that appears in the product uric acid molecule was confirmed by new kinetic data. It is concluded that this oxygen of the Very Rapid species does not, as frequently assumed, originate from the oxo group of the oxidized enzyme. A new turnover mechanism is proposed, not involving direct participation of the oxo ligand group, and based on that of Coucouvanis et al. [Coucouvanis, D., Toupadakis, A., Lane, J. D., Koo, S. M., Kim, C. G., Hadjikyriacou, A. (1991) *J. Am. Chem. Soc. 113*, 5271−5282]. It involves formal addition of the elements of the substrate (e.g., xanthine) across the Mo═S double bond, to give a Mo(VI) species. This is followed by attack of a "buried" water molecule (in the vicinity of molybdenum and perhaps a ligand of it) on the bound substrate carbon, to give an intermediate that on intramolecular one-electron oxidation gives the Very Rapid species. The latter, in keeping with the ^{13}C, ^{17}O, and ^{33}S couplings, is presumed to have the 8-CO group of the uric acid product molecule bonded side-on to molybdenum, with the sulfido molybdenum ligand retained, as in the oxidized enzyme.

Xanthine oxidase (Hille & Massey, 1985; Bray, 1988; Wootton et al., 1991; Hille, 1994) is the most extensively studied of molybdenum enzymes that depend on the pterin molybdenum cofactor (Rajagopalan & Johnson, 1992). It belongs to a group referred to as the molybdenum-containing hydroxylases (Bray, 1975). In addition to the molybdenum center, at which reducing substrates such as xanthine react, the enzyme contains FAD and iron−sulfur centers. The first three-dimensional structure from X-ray crystallography for an enzyme of this group, aldehyde oxidoreductase from *Desulfovibrio gigas*, has recently became available (Romão, et al., 1995).

Considerable information has accumulated about these enzymes, especially bovine milk xanthine oxidase, and their reaction mechanisms from spectroscopic studies, particularly by EPR and EXAFS (extended X-ray absorption fine structure) spectroscopies. After the reaction of reducing substrates at molybdenum, reducing equivalents are transferred intramolecularly from it to the flavin and iron−sulfur centers and the enzyme is reoxidized, via the flavin, by the oxidizing substrate, oxygen. Much attention has been directed toward understanding the enzymic reaction at molybdenum. In oxidized xanthine oxidase, the metal is in the Mo(VI) oxidation state, bearing an oxo- and a sulfido-ligand. Reaction with a substrate molecule, RH, reduces the metal to Mo(IV), and ultimately, the product, ROH (where the oxygen atom is derived originally from water) is liberated. The cycle is completed by reoxidation of molybdenum in two 1e$^-$ steps. EPR spectroscopic studies have revealed the existence of several clearly defined and structurally distinct Mo(V) reduced enzyme species. Appropriate substitutions

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* Authors to whom correspondence should be addressed.

‡ School of Chemistry and Molecular Sciences.

§ Present address: Laboratorio di Spettroscopia Molecolare, Dipartimento di Chimica, Università Degli Studi di Firenze, 50121 Firenze, Italy.

|| Nitrogen Fixation Laboratory; present address: John Innes Centre, Norwich, NR4 7UH, U.K.

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with stable isotopes have facilitated attempts to deduce the structures of these species, as well as investigations of their kinetic relationships and their hydrogen- and oxygen-transfer reactions (Bray, 1988). Current understanding (Bray, 1988; Turner et al., 1989; Pilato & Stiefel, 1993; Hille, 1994) of the catalytic mechanism of the enzyme depends in substantial measure on these studies, supplemented by studies of model compounds and model reactions (Holm, 1990; Coucouvanis et al., 1991; Greenwood et al., 1993; Enemark & Young, 1993).

Proposed mechanisms of the enzymic reaction depend crucially on the structure of a kinetically competent Mo(V) species known as "Very Rapid", in which the R residue of the substrate is bound to the enzyme. This species is observed when xanthine or certain other substrates are employed (Bray & George, 1985). Closely related to the Very Rapid species, in EPR parameters and therefore in structure, is the Alloxanthine Mo(V) species. This is the stable product of reaction of the reduced enzyme with the inhibitor, alloxanthine (Williams & Bray, 1981; Hawkes et al., 1984). Another species, named "Inhibited", represents the product of an inhibitory side reaction accompanying the turnover of formaldehyde or other aldehyde substrate molecules at the molybdenum centre (Pick et al., 1971; Morpeth & Bray, 1984).

ENDOR[1] spectroscopy (electron−nuclear double resonance) (Schweiger, 1982; Lowe, 1995) complements EPR in the analysis of ligand hyperfine couplings, being particularly valuable in the case of weak couplings. In a recent preliminary publication, Howes et al. (1994) reported ^{13}C-ENDOR spectroscopy studies of the Inhibited species. Mo−C distance estimates from dipolar coupling calculations based on the results provided evidence for a Mo−C bond in this species, the first direct evidence for such a bond in a biological system. We now describe ^{13}C- and ^{17}O-ENDOR studies that refine and extend structural information on the three molybdenum(V) species listed above. New fast-kinetic EPR and mass spectrometric studies of ^{17}O and ^{18}O exchange in the enzyme are also presented. Results provide evidence that molybdenum−carbon bond formation plays a part in the catalytic cycle and imply that earlier proposals that the enzyme operates by an oxo-transfer mechanism should be reconsidered. Preliminary reports on parts of this work have been presented (Bray et al., 1995, 1996).

MATERIALS AND METHODS

Xanthine Oxidase Samples. Bovine milk xanthine oxidase was from a commercial source (type XO2, from Biozyme Laboratories Ltd., Blaenavon, NP4 9RL, U.K.) but for a few experiments was prepared by the methods of Hart et al. (1970) or Ventom et al. (1988). Samples had A_{280}/A_{450} 5.0−6.0 and xanthine oxidase activities corresponding to 50−75% of the enzyme in the functional state[2] . SDS-PAGE of the Biozyme enzyme showed it to be largely nonproteolyzed, but the other preparations are extensively proteolyzed. No differences between the EPR properties of the preparations were noted (apart from those related to differing contents of the functional enzyme).[3] Highly concentrated samples for EPR and ENDOR spectroscopy were prepared in silica tubes of 4 mm internal diameter and frozen. Transfer of enzyme samples to ^{17}O- or ^{18}O-enriched H_2O was achieved by freeze-drying in 50 mM Na^+-bicine buffer, pH 8.2, and then redissolving the product in a minimum volume of enriched H_2O. Equilibration with the water was allowed to proceed for up to 20 h at 0 °C.

Specific molybdenum(V) EPR signals were generated as follows. The final concentration of the signal-giving species varied from about 60 μM for the xanthine Very Rapid signal to about 0.8 mM for the Inhibited signal. ^{13}C-[8]-Xanthine (Tanner et al., 1978; isotopic purity, 90%) was employed to generate the Very Rapid signal, using the rapid freezing procedure, with a reaction time of 13 ms at 22 °C, with 2.5 mM xanthine and 1.0 mM xanthine oxidase (total), in 50 mM carbonate buffer, pH 10.2 (Gutteridge & Bray, 1980). For the Very Rapid signal from 2-oxo-6-methylpurine, enzyme freeze-dried and equilibrated with ^{17}O-enriched H_2O was employed. The pH of the enzyme in enriched H_2O was raised shortly before the experiment by adding the solution to an excess of Na^+-Ches buffer, pH 10.1, that had been freeze-dried in an EPR tube and mixing gently in the tube until the buffer was dissolved. The EPR signal was developed in the tube, by the addition to the sample equilibrated with oxygen at 0 °C, of 2-oxo-6-methylpurine (0.9 mol/mol of functional enzyme). After mixing and a reaction time of 1 min, the sample was frozen in a melting isopentane bath (McWhirter & Hille, 1991). For some experiments, the enzyme was caused to turn over another substrate, prior to generation of the Very Rapid signal with 2-oxo-6-methylpurine. In this case, after the enzyme had been mixed with Ches buffer in the EPR tube, propionaldehyde (2.5 mol/mol of functional enzyme) was added under nitrogen. The sample was mixed and then occasionally stirred gently under oxygen for 1 h at 0 °C. Finally, 2-oxo-6-methylpurine (0.7 mol/mol of functional enzyme) was added as before to develop the Very Rapid signal.

For the Alloxanthine signal, freeze-dried enzyme equilibrated with ^{17}O-enriched H_2O was also employed. In this case no change of pH was required, and reduction with $Na_2S_2O_4$, addition of alloxanthine and oxidation with $K_3Fe(CN)_6$ in the presence of mediator dyes to give the signal-giving species, was carried out in the EPR tube according to Hawkes et al. (1984), monitoring the redox potential with electrodes in the tube (Cammack et al., 1988). Care was taken to ensure the absence of signals from reduced iron−sulfur centers.

The Inhibited signal was prepared with $^2H^{13}C^2HO$ (Cambridge Isotopes; enrichment: 99% ^{13}C, 98% ^{2}H). Xanthine oxidase (0.6 mM total) in 50mM Na^+-bicine buffer, pH 8.2, was treated with 0.2 M labeled formaldehyde for 1 h at 25 °C. The sample was then cooled, gel filtered to remove excess reagent, centrifuged, and concentrated overnight in the cold (Sartorius concentrator).

EPR and ENDOR Measurements. EPR spectra were recorded on a Bruker ESP300 instrument, with an NMR

[1] Abbreviation: ENDOR, electron−nuclear double resonance.

[2] "AFR" values at 23.5 °C [i.e., the rate of absorbance change in the standard assay with xanthine as substrate (Hart et al., 1970), divided by A_{450} for the enzyme sample] were 100−150 for the samples used. "AFR" values have been widely used (Bray, 1975) to estimate the percentage of the functional enzyme in xanthine oxidase preparations, relative to the content of the two nonfunctional forms, desulfo- and demolybdoxanthine oxidase.

[3] It is well-established [see e.g., Edmondson et al. (1972)] that only functional and not desulfo- or demolybdoxanthine oxidase gives EPR signals under the conditions used in the present work.

gaussmeter and a microwave frequency counter. Recording conditions were generally approximately as follows: modulation amplitude, 0.2 mT; microwave power 2−10 mW; temperature, 140 K. ENDOR spectra were recorded on an updated Bruker ER 200-SRC spectrometer equipped with an ENDOR/TRIPLE accessory and a radiofrequency amplifier of 100 W nominal output power (ENI 3100LA). Low temperature measurements were made using an Oxford Instruments ESR 900 cryostat modified to take sample tubes of up to 4 mm internal diameter. Recording conditions are specified in the figure captions. All ENDOR spectra were accumulated over extended periods of up to 48 h, to obtain adequate signal-to-noise ratios. It was essential, while working with the relatively weak ^{13}C and ^{17}O ENDOR signals, to subtract a background spectrum and other spurious features from the ENDOR spectrum. As these features tended to change with time, the ENDOR spectrum was recorded by alternating on and off resonance every 10 scans, using the Bruker automation routine. The resultant background (off resonance) spectrum was then subtracted from that on resonance. For spectral manipulation and simulation, data were transferred to a VAX computer.

Interpretation of Spectra. EPR spectra were interpreted with the help of computer simulations (Bray & George, 1985; George & Bray, 1988). ENDOR spectra were interpreted as described by Hoffman et al. (1984) and True et al. (1988). The samples used in the present study were frozen solutions, thus containing a random distribution of all protein orientations. ENDOR spectra recorded with the magnetic field set at the extremes of the EPR spectrum, however, give single-crystal-like patterns (Rist & Hyde, 1970). An ENDOR spectrum at an intermediate g value does not result from a single orientation but from a well-defined subset of molecular orientations. This has the effect that a magnetically equivalent ^{13}C set giving rise to only a pair of ENDOR peaks at the extremes of the EPR spectrum can display up to 12 ENDOR features at intermediate g values, as a consequence of the multiple orientations present at such g values. Therefore, instead of only a pair of peaks at positions defined as ν^+ and ν^-, one can expect an identical series of (up to six) peaks for ν^+ and ν^- (Hoffman et al., 1984). To simplify presentation, we generally show only the ν^+ series of peaks. The principal values of the hyperfine tensor and its orientation with respect to the g tensor frame can be determined by recording a series of ENDOR spectra across the EPR spectrum (Hoffman et al., 1984; True et al., 1988). This information provides a means of placing the nucleus in question into the local structure around the metal center. In some cases, the interpretation was facilitated by the use of a simulation program [based on the concepts of Hoffman et al. (1984)] which, however, had the limitation that it was unable to deal with significant admixture of molecular orientations by the hyperfine interaction. This is exemplified by the ^{13}C superhyperfine tensor of the Inhibited species for which we are, thus, unable to specify the Euler angles defining the relative orientation of the A- and g tensors.

Distance estimates from the dipolar contributions to ^{13}C-hyperfine couplings were made as follows. The main contributions to **A** are an isotropic term, A_{iso} and a dipolar term, A_D, given by

$$A_D = D\,(3\cos^2\Phi - 1)$$

where

$$D = g_e\beta_e g_n\beta_n/r^3$$

r is the unpaired electron-to-^{13}C-nucleus distance, and Φ is the azimuthal angle between the applied magnetic field and the vector joining the nucleus and the electron. The extreme values of A_D are 2D, when $\Phi = 0$ and $-D$, when $\Phi = 90°$. It follows that when $A_{iso} \neq 0$, the observed splittings are subject to the constraint $A_{||} + 2A_{\perp} = 3A_{iso}$.

This does not take account of the breakdown of the point dipole approximation for distances of the order of 2 Å [see, e.g. Schweiger, (1982)] and the effects of electron delocalization on to the carbon p-orbitals. Improved estimates may be obtained by calculating the point dipole interaction between the molybdenum and carbon nucleus using explicitly the molybdenum ground state d_{xy} orbital for the unpaired electron. However, not enough is known about the local structure for such a calculation to be feasible in the present case. The effects of electron density on the carbon p-orbitals are more amenable to calculation (Snetsinger et al., 1990). In addition to the dipolar superhyperfine interaction between molybdenum and carbons there are a number of other, generally small, interactions that may contribute to the 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 (Morton & Preston, 1978), 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 to give a corrected distance. This procedure was used to calculate corrected distances. However, the lack of a detailed local structure implies a degree of uncertainty in the resultant values.

For back-calculation of expected hyperfine anisotropies from assumed structures and associated likely bond-distances, simple dipolar coupling calculation was used, with further correction in the case of the Very Rapid signal for the substantial electron density delocalized from molybdenum on to sulfur (≈35%; George & Bray, 1988). It is thus necessary to take account of the superhyperfine interaction between the electron density on the sulfur atom and the xanthine C8 nucleus, in addition to that between the molybdenum and the C8 nucleus. Corrections were made for anisotropy arising from effects of putative Mo−C bonding using the measured A_{iso} to estimate the ^{13}C p-orbital electron density in the same way as for the corrected distance calculation above. The individual contributions of corrections to the calculated ^{13}C anisotropic hyperfine interactions are difficult to assess in the absence of precise information about the local structure and must therefore be regarded as approximate. For calculation purposes, typical interatomic separations were assumed and the maximum carbon anisotropic contributions, from electron density on the Mo [A_{aniso} (Mo)] and sulfur atoms [A_{aniso}(S)] only, were considered. Geometric arrangements in the different structures were assumed to be in the direction such as to bring calculated values of A_{aniso} closest to the experimental values.

Time Course of Exchange of ^{17}O out of Oxidized Xanthine Oxidase Monitored by EPR of the Very Rapid Signal. The experiment was carried out as described by Gutteridge and Bray (1980) using a multisyringe flow/mixing system. Samples of xanthine oxidase equilibrated with ^{17}O-enriched water and in 50 mM Na^+-bicine, pH 8.2, were diluted with the same buffer in ordinary water. After predetermined time intervals, progress of the exchange process was monitored by treating the samples with xanthine in 1 M carbonate buffer for 11 ms at a final pH of approximately 10.0, followed by rapid freezing and EPR of the Very Rapid signal. The proportion of ^{17}O in the signals was estimated from amplitudes of the ^{17}O hyperfine lines relative to those of the main lines.

Time Course of Exchange of ^{18}O Out of Oxidized Xanthine Oxidase Monitored by Mass Spectrometry of Enzymically Produced Uric Acid. Experiments were analogous to those of Hille and Sprecher (1987); however, our procedures (Turner, 1988) were developed independently of theirs and we used a flow/mixing system to ensure, where appropriate, that only the first turnover of the enzyme was studied. The flow system was analogous to that used in the experiment described in the previous subsection. As for that work, samples of xanthine oxidase, in 50 mM Na^+-bicine, pH 8.2, and equilibrated, in this case, with ^{18}O-enriched water, were diluted with ordinary water in the same buffer, and then, after predetermined time intervals, progress of the exchange was monitored. The procedure involved treating the enzyme with xanthine (approximately 1 mol/mol of active enzyme) for 83 ms and then squirting the xanthine/xanthine oxidase reaction mixture (1 vol) into acetone (2 vol) at about 50 °C to denature the enzyme. After centrifugation, uric acid was extracted from the supernatants by absorption on to an anion exchange resin (Dowex 2X-8), elution with dilute acetic acid, and freeze-drying and was derivitized by reaction with bis-(trimethylsilyl)trifluoroacetamide in the presence of dry pyridine. Gas chromatography/mass spectrometry was performed with a column of 3% OV-101 on Gas Chrom Q (Phase Separations Ltd.) at 240 °C, with a Kratos MS25 mass spectrometer, operated at an electron beam energy of 50 eV. Under the conditions used the tetrakis(trimethylsilyl)uric acid peak had a retention time of 3.5 min. Enrichment with ^{18}O was estimated from relative intensities of the peaks corresponding to *m/z* 456 and 458.

The system was tested by preliminary experiments using the flow system, in which ^{18}O-enriched xanthine oxidase samples, at a series of different concentrations, were treated briefly with xanthine (1.0 mol) in an excess of ordinary water. Enrichment of the uric acid obtained was found to increase as the quantity of xanthine oxidase was increased relative to xanthine. Under the conditions used, the maximum enrichment (corresponding to that before dilution) corresponded to a calculated $(m/z_{458})/(m/z_{456})$ ratio of 38.5% and the minimum enrichment (corresponding to that after dilution) to a ratio of 22.7%. The observed enrichments were 35.8% and 24.6% with 1.0 and 0.17 mol functional xanthine oxidase, respectively. Within this range we observed the linear relationship, between the uric acid enrichment achieved and the xanthine oxidase/xanthine ratio used, which may be predicted on the basis of uric acid being produced in the first turnover at the initial enrichment and in subsequent turnovers at the final enrichment (data not shown).

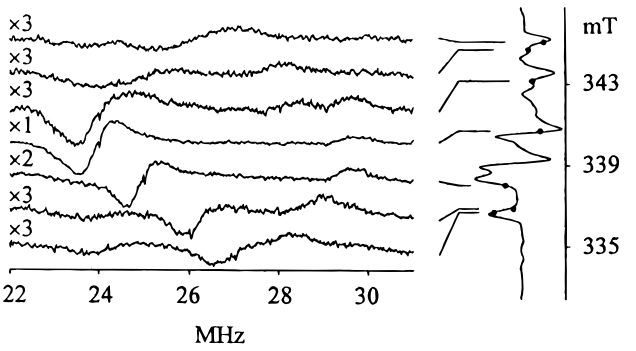

FIGURE 1: ^{13}C-ENDOR spectra from the Inhibited signal obtained by treatment of xanthine oxidase with $^2H^{13}C^2HO$ and recorded at field settings, as shown on the EPR spectrum plotted on the right, with the magnetic field scale indicated vertically. The relative amplification for each trace is given on the left. Recording conditions were as follows: temperature, 25 K; microwave frequency, 9.46 GHz; microwave power, 1 mW; RF modulation depth, 100 kHz; RF attenuation, 0dB; scan rate, 0.43 MHz s^{-1}.

RESULTS

^{13}C-ENDOR from the Inhibited Mo(V) EPR Signal. In a preliminary publication, Howes et al. (1994) presented a series of ^{13}C-ENDOR spectra recorded at intervals across the full EPR envelope of the xanthine oxidase Mo(V) Inhibited signal, generated with ^{13}C-labeled formaldehyde. This was done to analyze the ^{13}C-hyperfine tensor. However, the signal-to-noise ratio of some of the data was relatively poor. In the present work we improved the signal-to-noise ratios significantly by using formaldehyde labeled both with ^{13}C and ^2H, thereby obtaining an EPR spectrum consisting of single lines rather than doublets. Results are presented in Figure 1. Visual inspection of the ENDOR spectra (see Materials and Methods) enables the ^{13}C-hyperfine coupling tensor to be determined (Table 1), as outlined by Hoffman et al. (1984). [We were unable to determine the Euler angles relating the orientation of the hyperfine tensor with to that of the *g* tensor. However, it is clear from the behavior of the ^{13}C ENDOR features as a function of field that there is significant noncoincidence of the *A*- and *g* tensors; see Hoffman et al. (1984) and True et al. (1988)]. The ^{13}C-hyperfine tensor determined in the present work (Table 1) is identical to the one reported in our preliminary investigations (Howes et al., 1994).

Analysis of the anisotropic ^{13}C hyperfine tensor determined above in conjunction with the point dipole approximation can provide an estimate of the Mo−C distance in the Inhibited species (Table 2; see Materials and Methods). This gives a distance of 1.7 Å which may be corrected by the more sophisticated calculation to approximately 1.9 Å. It is recognized that such calculations do not yield fully reliable distance estimates for complexes where little is known about the metal−ligand molecular orbitals and the distance is less than about 2.0 Å. It was therefore of interest to use an alternative approach, back-calculating the predicted hyperfine anisotropies from various possible structures and their corresponding Mo−C distances. Results are given in Table 2, both for a structure suggested previously having a Mo−O−C−S ring arrangement (Turner et al., 1989) and for a Mo−C bond. The Mo−C distance consistent with the former is 2.6 Å (see Table 2 for references), which from the point dipole calculation, corrected for carbon p-orbital occupancy, would have an associated hyperfine anisotropy

Table 1: *g* Values and Superhyperfine Coupling Constants for Different Xanthine Oxidase EPR Signals[a]

signal	parameter	1	2	3	iso	α	β	γ	ref
formaldehyde Inhibited	*g*	1.9911	1.9772	1.9513					Tanner et al (1978)
	(A)^{13}C[b]	52.5	40.6	40.6	44.5				present work
	(A)^{17}O[c]	1.7	8.6	3.6					Bray and Gutteridge (1982)
		3.3	3.3	4.6					
	(A)^{33}S	1.7	0.8	5.5					Malthouse et al. (1981b)
xanthine Very Rapid	g	2.0252	1.9550	1.9494					Tanner et al. (1978)
	(A)^{13}C[d]	7.6	11.1	7.6	8.8	25	0	0	present work
	(A)^{17}O	38.0	38.3	37.1	37.8				Gutteridge and Bray (1980)
	(A)^{33}S[e]	8.5	76.6	19.1		40	0	10	Malthouse et al. (1981b)
2-oxo-6-methylpurine Very Rapid	g	2.0229	1.9518	1.9446					George and Bray (1988)
	(A)^{17}O[f]	32.4	34.7	35.4	34.2	30	0	0	present work
	(A)^{33}S	8.5	82.0	16.3		30	0	10	George and Bray (1988)
Alloxanthine	g	2.0279	1.9593	1.9442					Hawkes et al. (1984)
	(A)^{14}N	9.9	9.6	8.7					Hawkes et al. (1984)
	(A)^{17}O		not detected						present work
	(A)^{33}S[e]	8.5	85.0	19.0		50	0	10	Hawkes et al. (1984)

[a] Hyperfine coupling constants are given in MHz and angles of noncoincidence in degrees and were determined from the data as indicated in the text. [b] Euler angles α, β, and γ have not been determined, but from the experimental behavior clearly one or more is nonzero. [c] Bray and Gutteridge (1982) concluded, from EPR difference spectra obtained using two different enrichments of ^{17}O, that two weakly coupled oxygens with somewhat different parameters, as listed in the table, were present in the Inhibited signal-giving species. Signal-to-noise ratios were, however, poor, and more definitive work is required, e.g., using ENDOR. Two oxygens would be consistent with structure Ib (Figure 6) only if the oxo ligand had exchanged, which is not consistent (cf. Discussion) with our studies relating to the Very Rapid and Alloxanthine signals. [d] Parameters given are those that were used for the computer simulations shown in Figure 2. [e] Data recalculated with angles of noncoincidence as defined by George and Bray (1988). [f] Approximate values only; particularly, the Euler angles α, β, and γ have not been precisely determined.

Table 2: Mo−C Distances Calculated from ^{13}C-Superhyperfine Anisotropy of the Inhibited and Very Rapid EPR Signals: Comparison with Data Back-Calculated from Different Assumed Structures[a]

signal/assumed structure	A_{aniso}[b]	A_{iso}[b]	Mo−C distances (Å) (simple dipolar coupling calcn.)	Mo−C distances (Å) (corrected dipolar coupling calcn.)	Mo−C distances (Å) (assumed)
Inhibited (experimental data)	**11.9**	**44.5**	1.7	1.9	
Inhibited: assumed Mo−O−C−S ring structure	*3.3−6.3*				2.6[c]
Inhibited: assumed Mo−C structure	*7.5−10.5*				2.0[d]
xanthine Very Rapid (experimental data)	**3.5**	**8.8**	2.2	<2.15 or <2.35[e]	
Very Rapid: assumed Mo−O−C structure	*1.9−2.4*[f]				3.0−3.2[g]
Very Rapid: assumed Mo−C structure	*5.6−3.5*[h]				2.0[d]

[a] From the experimental data, the internuclear distances (normal type) were calculated from the experimental A_{aniso} values (bold type), using either simple dipolar or corrected dipolar calculation procedures, as described in Materials and Methods. From the various assumed structures and the corresponding likely internuclear distances (normal type), the expected anisotropic coupling values (in MHz) were calculated (italic type), using the simple dipolar coupling calculation, with corrections, as described in Materials and Methods. [b] Data from Table 1 are given in bold type; values of calculated A_{aniso} from assumed structures are in italics and include corrections for electron density of C p-orbitals and, for the Very Rapid species, delocalization on to S. For calculated A_{aniso} values, the uncorrected number is given first followed by the value obtained by assuming that all the corrections, discussed in Materials and Methods using the observed A_{iso}, add to give the calculated A_{aniso} value closest to the experimental $A_{aniso.}$ [d] Mo−C bond lengths, in complexes generally not relevant to the enzyme, vary from about 1.82 Å (Mo−C multiple bonds; Lemos et al., 1992) to 2.29 Å (Cotton et al., 1974). For some Mo-oxo complexes (Schrauzer et al., 1986; Hughes et al., 1994) they are in the range 2.02−2.21 Å. Mo−C distances in η^2-bonded acyl complexex of Mo(II) lie in the range 1.97−2.03 Å (Carmona et al., 1984; Durfee & Rothwell, 1988). Mo(IV) η^2-acyl complexes are known and although representative Mo−C distances have not been determined as yet, they are likely to be close to the W−C distance (2.00 Å) in the W analogue (Kreissl et al., 1987). [e] These two maximum distances depend on whether the electron density on the C orbitals is due to spin polarization or electron delocalization, respectively; electron density on sulfur is ignored but effects of this are not expected to be significant. [f] Assumes local structure such that the ^{13}C dipole tensors coupled with electron density on Mo and S sum to give the maximum possible resultant C A_{aniso}. [g] Mo−C distances in typical molybdenum complexes having a Mo−O−C structure [see, e.g., Walborsky et al., (1987) and Burrow et al., (1995)]. [h] For an assumed Mo−C distance of 2.1 Å, the values would be 4.8−2.7 MHz.

of <6.3 MHz. The measured anisotropy is 11.9 MHz, thus requiring an unreasonably large additional correction of 5.6 MHz to make a ring structure feasible. On the other hand, a Mo−C bond with an assumed distance of 2.0 Å would have a much larger associated corrected dipolar hyperfine anisotropy of up to 10.5 MHz, requiring a correction of only 1.4 MHz. Despite the uncertainties inherent in using the corrected point-dipole approximation to determine distances of the order 2.0 Å and the quite feasible possibility that the constraints of the enzyme on the active site may impose a shorter distance than that found for model compounds (viz. 2.0 Å), thus increasing the anisotropy expected, the agreement between the expected and the measured anisotropy is good. ^{13}C-ENDOR thus provides very strong evidence favoring a Mo−C bond in the Inhibited species, confirming the preliminary conclusions of Howes et al. (1994).

^{13}C-ENDOR from the Mo(V) Very Rapid EPR Signal. [8-^{13}C]Xanthine was used to generate the Very Rapid signal, and a series of ^{13}C ENDOR spectra were recorded across the EPR envelope (Figure 2a). [Note that a preliminary and rather different interpretation of ^{13}C data on the Very Rapid signal was given by Howes et al. (1991a)]. ENDOR simulations (Figure 2b) were performed as described in the Materials and Methods and gave an excellent fit to the data, thereby yielding highly reliable values (Table 1) for components of the ^{13}C hyperfine tensor, including the orientation

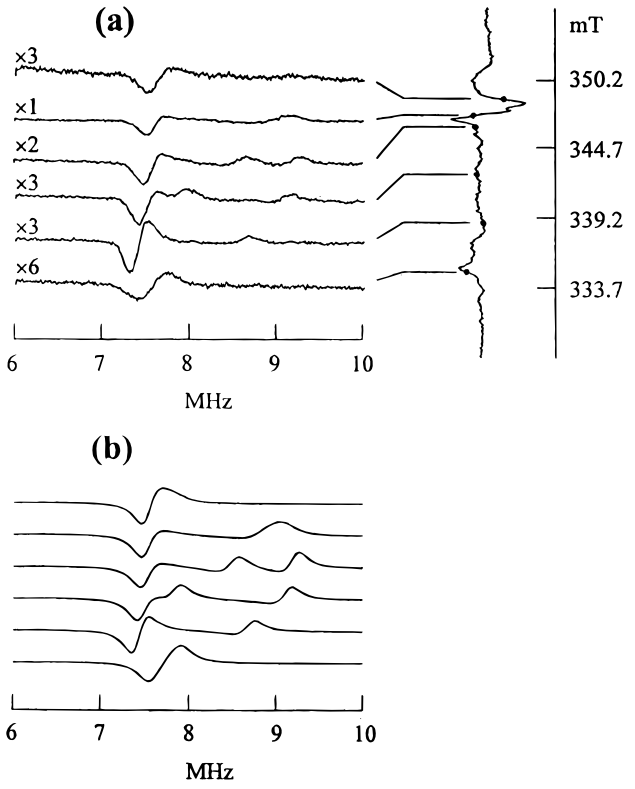

FIGURE 2: ^{13}C-ENDOR spectra from the Very Rapid signal obtained by reaction of xanthine oxidase with [8-^{13}C]xanthine; (a) experimental spectra and (b) computer simulations. Spectra in panel a were recorded at field settings, as shown on the EPR spectrum plotted on the right, with the magnetic field scale indicated vertically. The relative amplification for each trace is given on the left. Recording conditions were as follows: temperature, 30 K; microwave frequency, 9.46 GHz; microwave power, 2 mW; RF modulation depth, 100 kHz; RF attenuation, 0 dB; scan rate 0.43 MHz s^{-1}. The sample was prepared using rapid freezing. The simulations in panel b were computed as described in Materials and Methods by using the parameters given in Table 1. Field settings and approximate amplifications correspond to those in panel a.

of the *A* tensor with respect to the *g* tensor.

Estimation of the Mo−[8-C]xanthine distance using the point-dipole approximation (Table 2) is inherently more imprecise for the Very Rapid species than for the Inhibited species, as it is known that there is significant electron delocalization on to the sulfido ligand of the Very Rapid species (George & Bray, 1988). This results in two ^{13}C dipole tensors which sum in an uncertain way (depending on local structure) to give the measured anisotropic ^{13}C tensor. We were primarily interested in understanding whether the structure incorporates a Mo−[8-C]xanthine bond or if there is an intervening oxygen atom present. Therefore, we restricted our attention to back-calculations of the ^{13}C anisotropic hyperfine interactions resulting from electron density on the molybdenum and the sulfido ligand, to these two particular cases. Results of these calculations (Table 2) indicate that the measured anisotropy of 3.5 MHz is more likely to be consistent with a local structure that has a Mo−C bond than with bonding to the xanthine residue being via an oxygen ligand. However, it must borne in mind (see Materials and Methods) that, since we do not know the details of the local structure, the ^{13}C hyperfine tensors resulting from electron density on the molybdenum and sulfur atoms cannot be combined in a rigorous mathematical fashion.

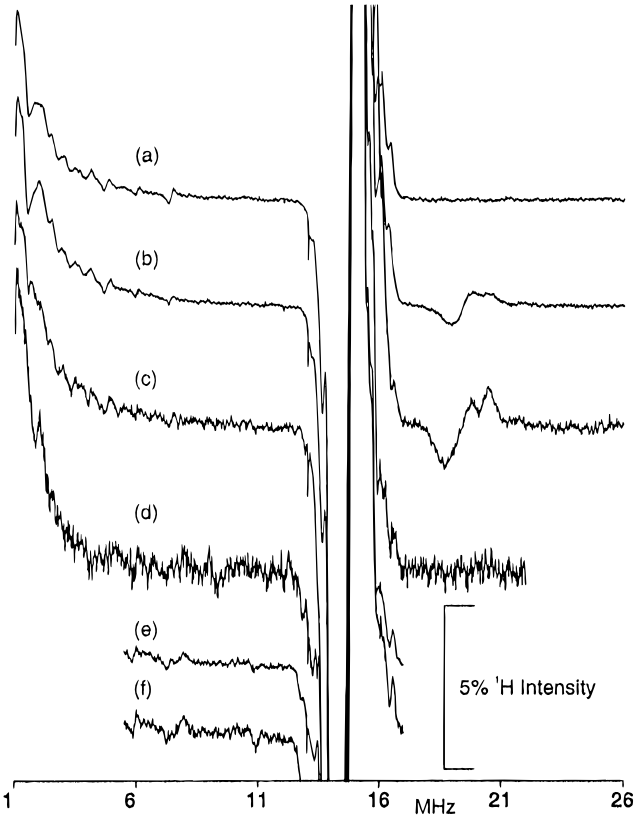

FIGURE 3: ^{17}O-ENDOR spectra from the Very Rapid and Alloxanthine signals obtained using [^{17}O]H_2O- equilibrated xanthine oxidase and ^{16}O controls. ENDOR spectra were recorded for samples showing the following EPR spectra: (a) Very Rapid, [^{16}O]-H_2O; (b) Very Rapid, [^{17}O]H_2O; (c) Very Rapid, [^{17}O]H_2O, after turnover; (d) Alloxanthine, [^{17}O]H_2O; (e) Alloxanthine, [^{16}O]H_2O; (f) Alloxanthine, [^{17}O]H_2O. Spectra were measured at a magnetic field corresponding to the peak of the EPR absorption spectrum. Recording conditions were as follows: temperature, 30 K; microwave frequency, about 9.42 GHz (depending on the sample); microwave power, 5 mW; RF modulation depth, 100 kHz at full power (nominally 100 mW). Scan rates were 0.30, 0.30, 0.30, 0.25, 0.27, and 0.27 MHz s^{-1}, respectively. The traces shown are the sums of 300, 200, 420, 850, 1940, and 1750 scans, respectively, with baselines subtracted. All spectra are normalized to give the same peak-to-peak intensity for the ^{1}H ENDOR signal (at 15 MHz), as shown by the marker. The Very Rapid signal was generated with 2-oxo-6-methylpurine; for trace c the enzyme was caused to turn over an aldehyde substrate in [^{17}O]H_2O prior to development of the signal (see Materials and Methods).

^{17}O-ENDOR of the Very Rapid Signal: The Strongly Coupled Oxygen and Attempted Detection of a Weakly Coupled Oxygen. To extend the EPR work of Gutteridge and Bray (1980) and George and Bray (1988), we studied ^{17}O-ENDOR spectra from the Very Rapid signal, obtained by treatment with 2-oxo-6-methylpurine, from xanthine oxidase equilibrated with [^{17}O]H_2O. We preferred this substrate to xanthine for this work because it gives rather stronger Very Rapid signals that may be obtained without the use of rapid freezing. Because ^{17}O-ENDOR is expected to be weak, it was important to obtain the strongest possible EPR signals. ENDOR spectra recorded at the peak of the EPR absorption and covering a wide radio frequency range are shown, for an enriched sample and an ^{16}O control, in Figure 3, traces b and a, respectively. Features due to the strongly coupled oxygen seen in EPR are clearly apparent around 19 MHz (with the low-frequency partner hidden under the proton ENDOR at around 15 MHz). Spectra recorded at different field settings across the EPR spectrum of the

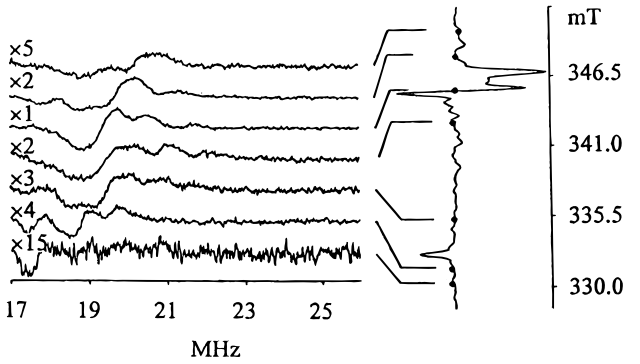

FIGURE 4: ^{17}O-ENDOR spectra from the Very Rapid signal obtained by treatment of [^{17}O]H_2O-equilibrated xanthine oxidase with 2-oxo-6-methylpurine and recorded at field settings, as shown on the EPR spectrum plotted on the right, with the magnetic field scale indicated vertically. The relative amplification for each trace is given on the left. Recording conditions were as follows: temperature, 30 K; microwave frequency, 9.44 GHz; microwave power, 5 mW; RF modulation depth, 100 kHz; RF attenuation, 0 dB; scan rate, 0.48 MHz s^{-1}.

^{17}O sample are shown in Figure 4. Parameters derived from the data are summarized in Table 1. These are in satisfactory agreement with those obtained by EPR (George & Bray, 1988). Simulations of the ENDOR data (not shown) were only partially satisfactory (due to the inability of our simulation program to deal with large hyperfine couplings) but clearly showed (a point not revealed by EPR) that the axes of the *g* and *A* tensors are noncoincident; they also provided an approximation (Table 1) to the angles of noncoincidence. Importantly, the data provide the new information that all components of the *A* tensor have the same sign. The estimated Mo-O distance from A_{aniso} 3.0 MHz (Table 1), allowing for 35% electron density on sulfur, is about 2.0 Å.

Earlier workers (Bray & George, 1985) in vain sought EPR evidence in the Very Rapid signal for a ^{17}O atom, weakly coupled to molybdenum, in addition to the strongly coupled one discussed above. In view of the potentialities of ENDOR for detecting weakly coupled ligands, we looked for ENDOR from a weakly coupled ^{17}O ligand in the above samples. However, the spectrum obtained (Figure 3b) did not differ significantly, below 13 MHz, from that of the control sample prepared with ordinary water (Figure 3a). In particular, no features centered on 2.02 MHz, the expected frequency for ^{17}O-ENDOR at the microwave frequency used, were apparent. [Note that the origin of some of the features, in the 1−8 MHz region and present in both the experimental and control samples (Figures 3a,b), is uncertain. Features at 6 MHz are presumably due to ^{31}P-ENDOR (Howes et al., 1991b), and an instrumental artifact gives rise to features at submultiples of the proton frequency, i.e., at 7.5 MHz, 5.0 MHz, etc.]. Thus, although a relatively strong nuclear quadrupole coupling of a hypothetical weakly coupled ^{17}O ligand would spread out its ENDOR features to such an extent that we might have been unable to detect them, it seems unlikely that one is present. Failure to observe features associated with a weakly coupled ^{17}O in the present (data not shown) and earlier EPR confirms this conclusion.

In further experiments we endeavored to extend, in comparison with earlier work (Bray & George, 1985), the range of conditions tried for exchange of a hypothetical weakly coupled oxygen ligand. In addition to examining samples, such as that used in Figure 3b and Figure 4 which had been equilibrated with ^{17}O-enriched water for 20 hr at 0 °C and pH 8.2, we examined another sample. Following similar equilibration with enriched water, this had turned over a small excess of an aldehyde substrate in enriched water prior to development of the Very Rapid signal. Such treatment would permit exchange of a second oxygen if this, like the strongly coupled one (Gutteridge & Bray, 1980), exchanged rapidly in the reduced enzyme. Results (Figure 3c) again show no evidence for a coupled ^{17}O atom, other than the strongly coupled one.

We conclude that, in the Very Rapid signal-giving species, it is very likely that the only oxygen atom in the vicinity of molybdenum that exchanges with water under the conditions of our experiments is the strongly coupled one studied previously.

Attempted Detection of a Coupled Oxygen in the Alloxanthine Signal by ^{17}O-ENDOR. The EPR parameters of the Alloxanthine signal are extremely similar to those of the Very Rapid signal (Table 1; see particularly *g* values, ^{33}S hyperfine splittings, and angular relationships). As already noted, this indicates a very close structural relationship between the two signal-giving species. The Alloxanthine signal (Hawkes et al., 1984) is not easy to study by EPR, owing to large line widths resulting from incompletely resolved ^{14}N-hyperfine structure, and to rather low conversion to the signal-giving species. When the Alloxanthine signal was prepared using enzyme equilibrated with enriched water, no ^{17}O-hyperfine coupling was detected by EPR by the above workers, a result confirmed in the present work (data not shown). The corresponding ENDOR spectra are shown in Figure 3. Enzyme equilibrated with ^{17}O-enriched water was used in Figure 3 traces d and f and in unenriched water for Figure 3e. The data clearly exclude the presence of a strongly coupled oxygen, which might conceivably have been missed in the work of Hawkes et al. (1984), in the Alloxanthine signal-giving species. Further, our new results provide no support for a more weakly coupled oxygen.

Time Courses of the Exchange of Oxygen Isotopes Out of Oxidized Xanthine Oxidase, As Monitored by Loss of ^{17}O Hyperfine Coupling in the Very Rapid EPR Signal or by Disappearence of ^{18}O from Uric Acid Produced in the First Enzyme Turnover. Hille and Sprecher (1987) used mass spectrometry to demonstrate, in single turnover experiments, ^{18}O transfer from the xanthine oxidase to uric acid, the product of xanthine oxidation. Using a rapid-mixing flow system, we extended such work to kinetic experiments on the exchange of ^{18}O out of the oxidized enzyme. These were carried out analogously to those on exchange of ^{17}O performed earlier by Gutteridge and Bray (1980), but using mass spectrometry of uric acid to follow the reaction, rather than EPR of the Very Rapid signal. Figure 5 shows the results of parallel experiments in which exchange of ^{18}O out of the oxidized enzyme was followed by mass spectrometry of uric acid and that of ^{17}O by EPR, as in the original work. Within the limits of the data, the exchange time courses followed by the two procedures are indistinguishible from one another.

Two additional points concerning Figure 5 call for comment. A possible partial explanation for data points at long reaction times falling below the theoretical infinite-time values might be provided by slow diffusion of ordinary water out of the nylon tubing into the samples. While the exchange rate constant derived from the data (approximately 3.5 h^{-1}

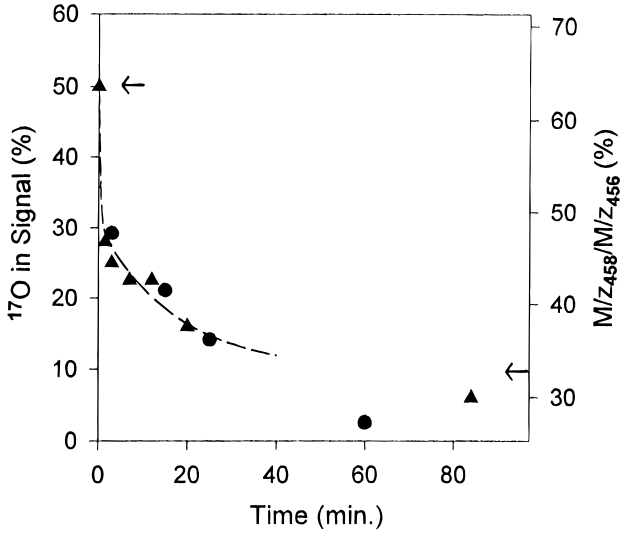

FIGURE 5: Time courses of the exchange of oxygen isotopes out of oxidized xanthine oxidase, monitored by loss of ^{17}O hyperfine coupling in the Very Rapid EPR signal or by disappearence of ^{18}O from uric acid produced in the first enzyme turnover. Xanthine oxidase was equilibrated with isotopically enriched water and then, at time zero, was diluted with ordinary water. After the time intervals indicated, the Very Rapid EPR signal was developed by treatment with xanthine for 11 ms, and the percent of ^{17}O in the signal was estimated (triangles) from the EPR spectrum. Alternatively, samples were treated with xanthine for 83 ms, the enzyme was denatured with hot acetone, and enrichment of the enzymically produced uric acid was estimated (circles) from mass spectra, following silylation and gas chromatography. The arrows correspond to calculated zero time and infinite time values in both experiments, based on the initial enrichments and the dilutions used. The experiments were carried out in 50 mM Na^+-bicine, pH 8.2, at 22 °C.

at 22 °C and pH 8.2) is fully consistent with the EPR results of Gutteridge and Bray (1980), according to Figure 5, there is apparently also an additional fast phase of the reaction. This was not apparent in the original work, but without further data it is premature to comment on its nature.

DISCUSSION

Evidence from ^{13}C-ENDOR for Mo−C Bonds in the Inhibited and Very Rapid Signal-Giving Species. Analysis of the dipolar coupling data, from the ^{13}C-hyperfine tensor obtained from ^{13}C-ENDOR, indicates Mo−C distances of about 1.9 Å in the Inhibited species and less than 2.4 Å in the Very Rapid species. In molybdenum complexes (whose structures generally have no specific relevance to that of the enzyme) Mo−C bond lengths vary from about 1.82 Å [Mo−C multiple bonds; e.g., Lemos, et al. (1992)] to 2.29 Å [Mo−C bonds, e.g., Cotton et al., (1974)]. The distance in the Inhibited species is thus within, and toward the middle of, the Mo−C bond length range, while for the Very Rapid species the distance is at the top of the range for such bonds. Furthermore, for both species back-calculations, from assumed structures of the expected hyperfine anisotropies, are consistent with Mo−C bonds.[4] Thus, the work provides strong evidence in favor of a Mo−C bond in the Inhibited species and suggestive evidence for one in the Very Rapid

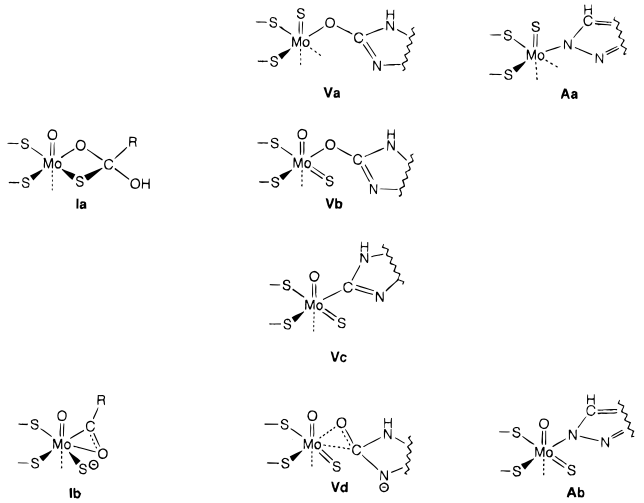

FIGURE 6: Possible structures of the different Mo(V)-signal-giving species. For the Inhibited signal-giving species, Ia is the structure suggested by Howes et al. (1990) and Ib (Howes et al., 1994) that favored in the present work. For the Very Rapid species, Va was suggested by Gutteridge and Bray (1980) and Vc by George and Bray (1988), though Vb was the structure favored by the latter workers; Vd is favored in the present work, with the oxygen bonded both to carbon and molybdenum being that detectable by EPR. For the Alloxanthine species, Aa was suggested by Hawkes et al. (1984) and Ab is favored in the present work. Note that, in all cases, additional ligands of molybdenum may be present and geometries may differ in detail from those illustrated.

species also. Earlier speculations on such bonds in xanthine oxidase species were dismissed (Bray, 1980; George & Bray, 1988; Turner et al., 1989) for the lack of positive evidence. It is noteworthy that this work provides the first direct evidence for a Mo−C bond in a biological system. However, such bonds may well also arise during reduction of acetylene and cyanide by nitrogenase. So far as we are aware, outside the cobalamines, it is also the first example of a bond in a functioning biological system between a carbon atom and a transition metal atom.

The Structure and Origin of the Inhibited Signal-Giving Species. Structure Ib (Figure 6) shows the structure now considered most likely for the Inhibited species, in comparison with a structure (Ia) proposed in earlier work (Turner et al., 1989; Howes et al., 1990). In structure Ib, the carbonyl group of the aldehyde substrate molecule is located side-on to the molybdenum and the aldehydic proton has been eliminated. Side-on Mo-(η^2-COR) structures are well-established among complexes of molybdenum in the oxidation states Mo(II) to Mo(IV) (Gambarotta et al., 1985; Carmona et al., 1984; Kreissl et al., 1987; Durfee & Rothwell, 1988).[5] Earlier EPR and EXAFS provide further support of structure Ib for the Inhibited species. EXAFS indicates (Turner et al., 1989) an oxo ligand on molybdenum.

[4] A limitation of our back-calculation approach is that, strictly, it is valid only in the absence of Mo−C bonding. Thus, strictly speaking, our results are inconsistent with the absence of Mo−C bonds and thus consistent with their presence.

[5] In a preliminary communication on the structure of the Inhibited signal-giving species (Howes et al., 1994), we quoted the structure $[P(C_6H_5)_4]_2[Mo(NO)\{\eta^2\text{-}OCN(CH_3)_2\}(NCS)_4]$ of Müller et al. (1979) to support our proposal of a "side-on"- or η^2-acyl structure. We learned subsequently from Prof. Müller that the above structure was in fact in error, a C and an N having been interchanged, and that more recently he has published (Müller & Mohan, 1981) a correct structure for a compound having the above formula. Unfortunately, this was published under the new name of the compound (a complex of an η^2-oximato ligand), and a correction has not yet been added to the crystallographic data base, so we did not realize that this error had occurred. We thank Prof. Müller for drawing our attention to it.

EPR with ^{17}O-treated formaldehyde in ^{17}O-enriched water (Bray & Gutteridge, 1982) showed weakly coupled oxygen atoms.[6] Furthermore, when the Inhibited signal is developed with formaldehyde (structure Ib, R = H), one formaldehyde proton is strongly coupled to molybdenum. This proton does not exchange with the solvent (Pick et al., 1971) and is not detected when the signal is developed with acetaldehyde or other aldehydes (Malthouse et al., 1981a).

As already noted, in structure Ib, the aldehyde carbonyl grouping has become bonded side-on to molybdenum. This is in accord with this signal being generated only by the action of aldehyde substrates. Structure Ib is not on the proposed catalytic pathway for the enzyme discussed below, consistent with the Inhibited species being on a side path.

Numbers and Nature of Oxygen Ligands of Molybdenum in the Very Rapid and Alloxanthine Species and the Identity of the Oxygen Transferred in Catalysis to the Product. A widely accepted mechanism for xanthine oxidase action has been advocated, with minor variations and in some cases with qualifications, by a number of workers (Gutteridge & Bray, 1980; Bray & George, 1985; Hille & Sprecher, 1987; Bray, 1988; Turner et al., 1989; Holm, 1990; Greenwood et al., 1993; Enemark & Young, 1993; Pilato & Stiefel, 1993; Hille, 1994; Lippard & Berg; 1994). This mechanism involves abstraction of the xanthine C8 proton by the sulfido ligand on molybdenum, to give an -SH structure, with concerted attack by the oxo ligand on the substrate C8 carbon to give an $Mo^{IV}-O-C$ species. Intramolecular $1e^-$-oxidation (by the flavin and iron−sulfur centers) then gives the Very Rapid species. Completion of the catalytic cycle involves (not necessarily in this order) reoxidation of molybdenum, fission of the Mo−O bond with the oxygen becoming that of the uric acid product, and regeneration of the oxo ligand from water. We consider below, in the light of this proposed mechanism, the new and earlier data on oxygen ligands in the various species.

There is clear and direct evidence in the literature for oxygen ligands on molybdenum. In the oxidized enzyme, a single oxygen ligand as an oxo group is well established from EXAFS and is retained in all reduced enzyme forms so far studied by this technique (Bordas et al., 1980; Cramer et al., 1981; Cramer & Hille, 1985; Turner et al., 1989; Hille et al., 1989). The single strongly coupled oxygen detected by EPR in the Mo(V) Very Rapid species was considered unlikely to be an oxo group by Gutteridge and Bray (1980), though later workers (George & Bray, 1988) considered this possibility not to have been fully excluded. For the alloxanthine complex, in the Mo(V) state (Alloxanthine species), Hawkes et al. (1984) were unable to detect hyperfine coupling to oxygen in the EPR spectrum. On the other hand, both Turner et al. (1989) and Hille et al (1989) found evidence for an oxo ligand on molybdenum in EXAFS of the alloxanthine enzyme complex in the molybdenum(IV) state. This suggests very strongly that such a group must also be present in the Mo(V) Alloxanthine signal-giving species, despite the failure of EPR to detect it. Indeed, it is not easy to see how the oxo group of the oxidized enzyme might have been eliminated in enzyme reduction and complex formation with alloxanthine, since the oxo is retained in the reduced enzyme, and this inhibitor is not transformed in any way by the enzyme (Massey et al., 1970). The expectation of an oxo ligand in the Mo(V) Alloxanthine species leads in turn to an expectation that such a ligand should be present, also, in the Very Rapid species. This follows since, as already discussed, the EPR parameters for the two species are very close to one another, so that their structures must be very similar.

Specific data in the literature concerning the absence of oxygen ligands are as follows. $[^{17}O]H_2O$-equilibrated enzyme was employed in the earlier EPR work in which the single strongly coupled oxygen ligand was detected in the Very Rapid species (Gutteridge & Bray, 1980; Bray & George, 1985). However, the possibility that there might be a second oxygen ligand in this species has frequently been mooted and variants of the above catalytic mechanism imply that one should be present. Thus another, more weakly coupled, ^{17}O ligand was sought but not found by EPR by George and Bray (1985), by extending up to 20 days the period allowed for equilibration of the enzyme with ^{17}O-water. For the Alloxanthine species, Hawkes et al. (1984) were unable, using $[^{17}O]H_2O$-equilibrated enzyme, to detect any ^{17}O-hyperfine coupling. The difficulies of EPR work with this species have, however, been noted.

Against this background, we used ^{17}O-ENDOR to investigate further the nature and numbers of oxygen ligands in the Very Rapid and Alloxanthine species. For the Very Rapid species, our data fully confirm the presence of the strongly coupled oxygen detected by EPR. Furthermore, by showing for the first time that the components of the hyperfine coupling tensor all have the same sign, the data further diminish any possibility (George & Bray, 1988) that the coupled oxygen might be an oxo ligand of molybdenum. For the Alloxanthine species the ^{17}O-ENDOR confirms and extends the conclusion of Hawkes et al. (1984) that no coupled oxygen can be detected. These workers used higher derivative spectra, a technique most appropriate to seeking a weakly coupled oxygen. It seemed possible that a relatively strong ^{17}O-coupling might have been missed in the original EPR work. Simulations (not shown) indicate that, with particular values of the couplings not greatly different from those for the Very Rapid species, ^{17}O-hyperfine EPR lines from the Alloxanthine species would merge together and thus become very difficult to detect. However, the ENDOR data make it clear that no oxygen atom with a hyperfine couping constant comparable to that for the Very Rapid species is present in the Alloxanthine species. The Alloxanthine and the Very Rapid species are expected to have in common an oxo ligand. However, clearly a strongly coupled oxygen exchangeable in the oxidized enzyme is something they do not have in common. This result thus provides final confirmation that the strongly coupled oxygen of the Very Rapid species is not an oxo group.

Though the ^{17}O-ENDOR data in the low-frequency region, particularly for the Alloxanthine species, are not definitive, they provide no evidence for an additional more weakly coupled oxygen that has exchanged under the conditions of our experiments in either the Alloxanthine or the Very Rapid species.

Our finding that the exchange time course for oxygen out of the oxidized enzyme is identical when followed by two different procedures is also relevant to the possibility that the molybdenum atom of the Very Rapid species might bear

[6] See footnote c, Table 1.

more than one oxygen ligand. The data provide the strongest evidence to date that a specific oxygen atom, present in the vicinity of molybdenum in the oxidized enzyme, is both the oxygen "seen" in the Very Rapid signal and the one transferred to the uric acid product. That this is the case has hitherto been tacitly assumed (Hille & Sprecher, 1987). This conclusion was presumably based largely on these workers' qualitative finding that the transferred oxygen exchanged faster in the reduced than in the oxidized enzyme, by analogy with the quantitive earlier kinetic data on oxygen exchange, as followed via the Very Rapid signal by Gutteridge and Bray (1980).

The data described so far present something of a paradox. On one hand, from the Very Rapid species no EPR or ENDOR evidence has been obtained for more than a single oxygen ligand, with the one detected now confirmed not to be an oxo group, and there is no evidence from earlier EPR or the present ENDOR for any oxygen ligand in the Alloxanthine species. On the other, results from EXAFS and expectations from the proposed reaction mechanism make it very clear that an oxo ligand should be present in both these species.

The paradox disappears if the long-standing notion (Gutteridge & Bray, 1980; Hille & Sprecher, 1987; Holm, 1990), that the terminal oxo ligand of the oxidized enzyme is the group involved in exchange studies and the catalytic reaction, is abandoned. Perhaps surprisingly, this view has been almost universally accepted in the literature since it was introduced. Holm (1990) concluded that oxo transfer was the "most reasonable interpretation" of the data and others [eg., Lippard and Berg (1994)] followed this interpretation. However, Pilato and Stiefel (1993) point out that there are other possibilities. More specifically, Hille (1994), while continuing to regard oxo group involvement as most likely, raises the alternative that a hydroxyl group might be the ligand involved instead.

In model compounds, exchange rates for oxygen ligands of molybdenum vary from a half-time of seconds (von Felten et al., 1978) to years (Rodgers et al., 1985). As pointed out by Bray (1988), the exchange rate in the enzyme is consistent (Murmann, 1980) with an oxo group in the oxidized enzyme, provided (Johnson & Murmann, 1983) a coordination site for water is available *cis* to the oxo. However, other explanations need to be considered. Though water or hydroxyl ligands of molybdenum generally exchange rapidly in low M_r complexes, this situation need not necessarily hold in the enzyme, where access to the active site may be restricted. We now propose, and develop further below, the hypothesis that the exchangable oxygen in the oxidised enzyme is a "buried" water molecule or a hydroxyl ion that is in the vicinity of the molybdenum atom and quite possibly ligated to it. This hypothesis could account, as readily as the oxo group hypothesis, for oxygen exchange becoming fast in reduced enzyme, provided it is assumed that access to the Mo center is less restricted in the reduced than in the oxidized enzyme.

Structures of the Very Rapid and Alloxanthine Species and the Mechanism of Reaction of Reducing Substrates with the Molybdenum Center. Possible structures of the various signal-giving species are illustrated in Figure 6. For the Very Rapid species, structure Va was first suggested by Gutteridge and Bray (1980), and structure Aa is its analogue for the Alloxanthine species (Hawkes et al., 1984). We reject these structures, since they include no oxo group. Structures Vb and Vc were both regarded as more likely than Va for the Very Rapid species by George and Bray (1988). We reject Vc on the grounds that we cannot conceive of any reaction mechanism that would give rise to it. Structure Vb, widely accepted for the Very Rapid species, suffers from the disadvantage that it is not compatible with the molybdenum−carbon distance estimates of the present work. A further inadequacy concerns the enzymic mechanism discussed above. According to this, the oxo ligand of the oxidized enzyme becomes the Mo−O−C group of the Very Rapid species. Thus, in structure Vb, the oxo group is not the original one but has been regenerated by water or hydroxyl attack. If this were the case, when the reaction is carried out in ^{17}O-water, both oxygens should be detected. As already discussed, neither the present nor earlier work provides any support for the presence, under any conditions used, of a second coupled oxygen that might correspond to the oxo group. Note that even in an axial position, an oxo ligand is expected to give a ^{17}O coupling that should be readily detectable by EPR or by ENDOR. Thus, for example, the complex $[MoO(SPh)_4]^-$ shows A(^{17}O) 8.6, 8.6, 1.9 MHz (Hanson et al., 1987). Conversely, in the Alloxanthine species (taking structure Ab as the analogue of structure Vb), if the oxo group is exchangeable, it should also give detectable ^{17}O coupling, but no support for its presence has been found in the present ENDOR or in earlier EPR (Hawkes et al., 1984). Thus, structure Vd is the one we now favor for the Very Rapid species. For the Alloxanthine signal-giving species, we favor stucture Ab [originally proposed by Turner et al. (1989)] since it may be considered analogous to structure Vd.

Other data supporting structures Vd and Ab for the Very Rapid and Alloxanthine species are as follows. In comparison with other Mo(V) species from xanthine oxidase, these show strong coupling (Malthouse & Bray, 1980; Malthouse et al., 1981b; Hawkes et al.,1984; George & Bray, 1988) to the sulfur atom that is cyanide-labile in the oxidized enzyme. Furthermore, and again in contrast to other xanthine oxidase species, the ^{97}Mo nuclear quadrupole coupling tensor in the Very Rapid species is highly anisotropic (George & Bray, 1988). These findings argue for a structure substantially different from other xanthine oxidase species and are the primary justification for our conclusion that the sulfido ligand is retained. In structure Vd, note that the orientation of the side-on CO is shown reversed in relation to that in the Inhibited species (structure Ib). This is consistent with the observed reversal (Table 1) of the relative magnitudes of the hyperfine couplings to oxygen and to carbon in the two species. The approximate Mo−O distance (∼2.0 Å) from our ^{17}O ENDOR is also consistent with structure Vd, as is the Mo−^{2}H distance of about 3.2 Å, reported from pulsed EPR for a deuteron in the N7 position of the 2-oxo-6-methylpurine residue (Lorigan et al., 1994).

Wilson et al. (1991) proposed the compound $[MoOSL]^-$, where L = *N*,*N*′-dimethyl-*N*,*N*′-bis(2-mercaptophenyl)-1,2-diaminoethane, as a model for the Very Rapid species, and it is a good one so far as ^{33}S coupling is concerned. However, ^{17}O coupling in $[MoOSL]^-$ is weak (Greenwood et al., 1993), so that it is a poor model for ^{17}O couplings. This complex, which lacks any analogue for the bound xanthine residue, is thus confirmed to be an inexact model for the Very Rapid species.

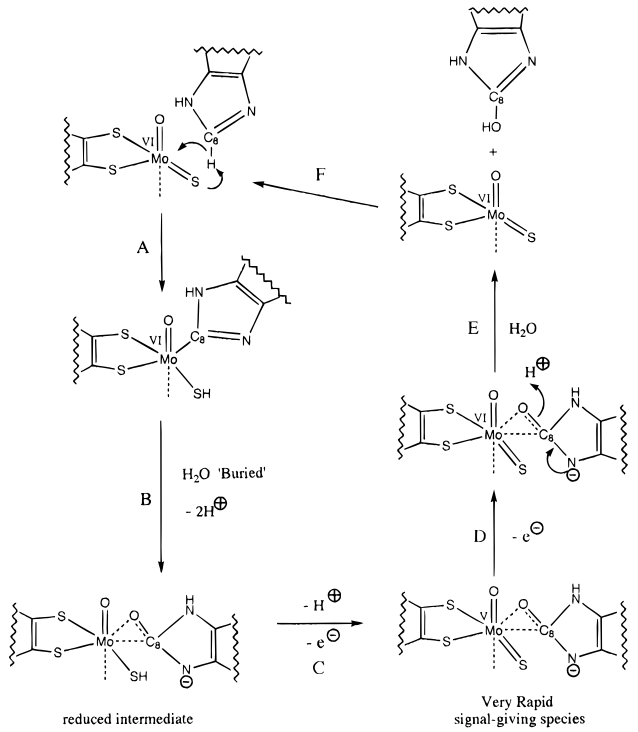

FIGURE 7: Proposed catalytic cycle for xanthine oxidation by xanthine oxidase. The nature of reaction steps A−F is discussed in the text. Note that the possibility is not excluded that, in a parallel pathway, product dissociation occurs prior to molybdenum reoxidation. Amino acid residues participating in the reactions are not included.

Our proposal for the reaction mechanism of xanthine oxidase, based on and further supporting structure Vd for the Very Rapid species, is illustrated in Figure 7 and involves abandonment of the notion of an oxo-transfer mechanism for the enzyme. Rather, it is better categorized as an "oxygen atom insertion" (Pilato & Stiefel, 1993). As with the earlier mechanism, the scheme is heavily dependent on the retention in the Very Rapid species of the xanthine residue, as originally demonstrated by Tanner et al. (1978) by EPR using ^{13}C-labeled substrate and as now further studied by ^{13}C-ENDOR. It also depends on retention in this species of the oxygen atom that exchanges slowly in the oxidized enzyme, as first studied by Gutteridge and Bray (1980). Our exchange studies make more rigorous the assumption of Hille and Sprecher (1987) that it is this oxygen that ultimately becomes that of the uric acid product. This oxygen is now proposed as originating from a "buried" water rather than as an oxo group. Note that the oxo molybdenum ligand now plays no active part in the reaction. Again, as with earlier mechanisms, that of Figure 7 is also supported by EPR kinetic evidence [Gutteridge et al., 1978; see also Bray (1988)] for transfer of the substrate C-8 proton to the sulfido ligand of molybdenum. As discussed above, the original mechanism involved addition of the xanthine residue to the oxo ligand and of the C-8 proton to the sulfido ligand. Evidence for a precursor of the Very Rapid species, assumed to be in the Mo(IV) state, has been provided by McWhirter and Hille (1991). Our new mechanism involves (Figure 7, step A) formal addition of the elements of the substrate molecule, RH, across the Mo═S double bond, to give a Mo(VI) species. Presumably this would occur by concerted electrophilic attack by molybdenum on the C8 carbon and proton abstraction by the sulfido group. Such a step was suggested, but not favored, by Turner et al. (1989), on the basis of the *ab initio* calculations of Rappé and Goddard (1982). More recently, Coucouvanis et al. (1991) have provided more direct support from the reactions of model compounds and have indeed suggested a mechanism for xanthine oxidase action closely related to that proposed here. Step B of Figure 7 involves attack by the "buried" water (quite possibly via conversion to a hydroxyl ligand on molybdenum) on the molybdenum-bound carbon, resulting in reduction of the metal and formation of a side-on molybdenum CO species. In step C, transfer of an electron from molybdenum to iron−sulfur or flavin and loss of a proton gives the Very Rapid species. The catalytic cycle is completed (steps D−F) by product dissociation, following transfer of a second electron from molybdenum to iron−sulfur and uptake of a proton.

The mechanism as depicted gives no indication of the part played by amino acid residues and is thus obviously an oversimplification. Structural work reported to date has provided limited information concerning the molybdenum environment. However, a glutamate residue, Glu-869 in *D. gigas* aldehyde oxidoreductase, that is conserved in all enzymes of this group is close to molybdenum (Romão et al., 1995) and could play a role in proton transfer.

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