Studies by Electron Paramagnetic Resonance Spectroscopy of Xanthine Oxidase Enriched with Molybdenum-95 and with Molybdenum-97†

Graham N. George* and Robert C. Bray

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

Received December 21, 1987

ABSTRACT: Investigations have been carried out on the nature of the species from the enzyme xanthine oxidase that give rise to two molybdenum(V) electron paramagnetic resonance (EPR) signals. Isotopic enrichment with ^{95}Mo, ^{97}Mo, ^{33}S, and ^{17}O was employed. Computer simulations of the EPR spectra recorded at 9- and 35-GHz microwave frequencies were used to evaluate the various hyperfine couplings and angular relations between the principal axes of **g** and **A**, as well as the nuclear electric quadrupole interaction for ^{97}Mo. The results support the presence of an oxo ligand in the Rapid and of both an oxo and a sulfido ligand in the Very Rapid signal-giving species.

Xanthine oxidase catalyzes the hydroxylation of a wide range of purines, aldehydes, and other organic componds in a complex process that involves the molybdenum, the flavin, and the two different iron–sulfur sites which are part of the enzyme molecule (Bray, 1975, 1988; Hille & Massey, 1985). The generally accepted overall reaction mechanism is as follows: the substrate (e.g., xanthine) is oxidized at the molybdenum site, which is reduced from Mo(VI) to Mo(IV) during the process. A rapid equilibration of the reducing electrons then occurs with the other redox active sites of the enzyme (Anderson et al., 1986), enabling reoxidation of the enzyme to occur by reduction of oxygen at the flavin site. The enzyme can accept a total of six electrons from three reducing substrate molecules, two being accepted by the molybdenum, two by the flavin, and one by each of the two iron–sulfur clusters. The redox potentials of the molybdenum, flavin, and iron–sulfur sites are such that for less than fully reduced enzyme molecules (i.e., those that have accepted one to five electrons) appreciable amounts of paramagnetic Mo(V) is present, which can be detected by its characteristic $S = {}^1/_2$ EPR[1] spectrum. A large number of different Mo(V) EPR signals are known for xanthine oxidase (Bray, 1980, 1988), and EPR spectroscopy, together with EXAFS spectroscopy, has been a major contributor in the current understanding of the catalytic reaction occurring at the molybdenum site.

EXAFS investigations (Bordas et al., 1980; Cramer et al., 1981) reveal that the fully oxidized [Mo(VI)] site bears an oxo (Mo=O) and a sulfido (Mo=S) ligand, together with a number (two or three) of thiolate type (Mo–SR) groups at longer bond distances from the metal. Upon reduction to Mo(IV) a terminal sulfido is no longer observed (Cramer et al., 1981) presumably [cf. Gutteridge et al. (1978)] because of protonation to form an Mo–SH group. These conclusions are fully consistent [cf. Bray (1988)] with the known chemistry of molybdenum complexes. The Mo=S group of oxidized enzyme is of particular interest; it can be removed by reaction with cyanide to form thiocyanate. The inactive desulfo enzyme, possessing an extra Mo=O group, that is produced in this reaction can be reconstituted to yield functional enzyme by reaction with sulfide (Massey & Edmondson, 1970; Gutteridge et al., 1978; Bray, 1988).

The Mo(V) EPR signal called Very Rapid is the first Mo(V) signal observed after xanthine oxidase is mixed with the purine substrate xanthine (Bray, 1980; Bray & George, 1985). It reaches maximum intensity at about 10 ms after initiation of the reaction and then decays more slowly within the turnover time. A second EPR signal, called Rapid, is also detectable during turnover. Kinetics of its appearance are characterized under some conditions, and in contrast to those of the Very Rapid signal, by a brief lag phase. The Rapid signal is also obtained in partially reduced enzyme samples at equilibrium. It is thought to correspond (Bray, 1980, 1988) to the usual reduced form of the functional enzyme. The Very Rapid signal, on the other hand, is thought to represent an intermediate of turnover in which the substrate carbon that is to be hydroxylated is covalently associated with molybdenum (Tanner et al., 1978; Gutteridge & Bray, 1980).

Extensive studies have been carried out (Bray, 1980, 1988; Bray & George, 1985) in efforts to establish the nature of the various Mo(V) EPR signal-giving species from xanthine oxidase. Particularly important in these have been substitution studies with a range of stable isotopes. Despite the wealth of EPR spectroscopic data upon the Very Rapid signal, there still remain [cf. Bray and George (1985)] some open questions as to the structure of the signal-giving species, and its exact relation to the catalytic mechanism.

Analysis of the hyperfine couplings from ^{95}Mo and ^{97}Mo, and of nuclear quadrupole couplings to the latter nucleus, is in principal capable of yielding important structural information on molybdenum in enzymes. Xanthine oxidase was enriched in ^{95}Mo as long ago as 1966, and spectra of the Mo(V) EPR signals from such samples were recorded (Bray & Meriwether, 1966), but proper analysis of complex spectra, such as these, is not possible without computer simulation of the powder line shape [cf. George and Bray (1983) and Seebauer et al. (1983)]. We now report detailed studies on xanthine oxidase enriched with these isotopes, in which par-

† This work was supported by grants from the Science and Engineering Council, the Medical Research Council, and the Agricultural and Food Research Council.

* Author to whom correspondence should be addressed at Exxon Research and Engineering Co., Clinton Township, Route 22E, Annandale, NJ 08801.

[1] Abbreviations: EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption edge fine structure.

0006-2960/88/0427-3603$01.50/0 © 1988 American Chemical Society

ticular attention was paid to the Very Rapid signal.

Materials and Methods

Preparation of Enzyme Samples. Xanthine oxidase was prepared from fresh bovine buttermilk according to the salicylate denaturation procedure (Hart et al., 1970; Bray, 1982). Concentrations of the functional enzyme were calculated according to Bray (1975). The molybdenum isotopes, obtained as the metal (from Oak Ridge National Laboratory, Oak Ridge, TN 37831), were converted to 0.11 M solutions of Na_2MoO_4 by dissolving in excess nitric acid, evaporating to dryness, and dissolving in a stoichiometric quantity of aqueous NaOH. The solutions were injected intravenously into the jugular veins of cows. Milkings from 19 to 76 h after injection were taken. The enzyme was prepared from the pooled cream from four to six cows. Injection of 0.30 and 0.40 mg of Mo/kg of body weight, for ^{97}Mo and ^{95}Mo, respectively, yielded enzyme enriched 70–75% in ^{97}Mo and 80–85% in ^{95}Mo, as determined by EPR.

Enrichment of the enzyme with ^{17}O was carried out according to Bray and Gutteridge (1982). Enzyme enriched in the cyanide-labile site with ^{33}S was prepared according to Malthouse and Bray (1980). Samples for EPR spectroscopy were prepared in quartz tubes of 3- or 2-mm internal diameter for X- and Q-band measurements, respectively. Final concentrations of functional active centers were 0.2–0.5 mM.

The xanthine Very Rapid signal was prepared by the rapid freeze technique according to Gutteridge and Bray (1980). The 2-oxo-6-methylpurine Very Rapid signal (Bray & George, 1985) was prepared by manual mixing and so gave better signal-to-noise ratios. An excess of substrate (ca. 10 mM) was added to enzyme contained in an EPR tube, oxygen was bubbled through the sample for a few seconds, and it was immediately frozen by immersion in cold isopentane. The formamide Rapid signal was prepared according to Morpeth et al. (1984).

EPR Spectroscopy. EPR spectra were recorded on a Varian E9 instrument interfaced to an ACT Apricot Xi computer. A dual sample microwave cavity was used for X-band measurements, with simultaneous recording of experimental and Mn^{2+} – diphenylpicrylhydrazyl reference spectra on to the computer, so as to permit accurate field alignment, as described by Bray et al. (1978). Some spectra were recorded on an earlier computer system (Bray et al., 1978). EPR spectra were recorded at a temperature of 123 K with nonsaturating microwave powers. Modulation amplitudes of 0.16 and 0.25 mT were used for X-band and Q-band measurements, respectively. For manipulation and simulation, spectra were transferred to the University of Sussex DEC VAX 11-780 computer.

Computer Procedures and Computer Simulation of EPR Spectra. EPR spectra corresponding to 100% enrichment of ^{95}Mo, ^{97}Mo, ^{33}S, or ^{17}O were obtained from the experimental spectra by computer subtraction of the appropriate amount of the spectrum of unenriched (natural abundance) enzyme.

EPR spectra of ^{95}Mo-, ^{33}S-, and ^{17}O-enriched samples were fitted to the $S = {}^1/_2$ spin Hamiltonian:

$$H = \beta \mathbf{H}\cdot\mathbf{g}\cdot\mathbf{S} + h\mathbf{S}\cdot\mathbf{A}\cdot\mathbf{I} \qquad (1)$$

Software, based upon a second-order perturbation solution of eq 1, was employed that permitted iterative computer minimization of the error between experimental and calculated curves, together with alteration of parameters in an interactive manner by the user. Additional sets of hyperfine coupling were included to first order where necessary. For simulation of EPR spectra of ^{97}Mo-enriched samples, the 11.5-fold larger nuclear electric quadrupole coupling expected (Blumer et al., 1973) for this isotope in comparison with that of ^{95}Mo required a more rigorous approach. In this case the spectra were fitted to the Hamiltonian

$$H = \beta \mathbf{H}\cdot\mathbf{g}\cdot\mathbf{S} + h\mathbf{S}\cdot\mathbf{A}\cdot\mathbf{I} + h\mathbf{I}\cdot\mathbf{P}\cdot\mathbf{I} - \beta_N g_N \mathbf{H}\cdot\mathbf{I} \qquad (2)$$

A modified version of the program QPOW of R. L. Belford and co-workers (Seebauer et al., 1983) was used. This program diagonalizes the spin Hamiltonian matrix, to afford very accurate simulations. The much larger amounts of computer time used by this program meant that computer minimization of errors was not practicable, and spectra were generally fitted by inspection, although the sum of the squares of residuals between calculated and experimental curves was still used as a criterion of correctness of fit. Values of –0.3654 and –0.3731 were used for the nuclear g values of ^{95}Mo and ^{97}Mo, respectively. Simulations of the ^{95}Mo data with QPOW gave results that were indistinguishable from those from the perturbation solution program.

The sequence of operations used in fitting the ^{95}Mo and ^{97}Mo spectra was as follows. The parameters of $I = 0$ features of the spectra from unenriched samples were refined by fitting, starting from literature values, and these parameters were then fixed for subsequent simulations of the spectra of enriched samples. $\mathbf{A}(^{95}\text{Mo})$ was then obtained from simulations with the program based on the perturbation solution. $\mathbf{A}(^{97}\text{Mo})$ was then calculated from $\mathbf{A}(^{95}\text{Mo})$ and the ratio of the nuclear g factors. Thus only the quadrupole coupling needed to be varied in the ^{97}Mo simulations.

The criterion of an accurate fit was that a single set of parameters (except line width) should fit both X- and Q-band data well.

Results

^{95}Mo and ^{97}Mo Isotopic Substitution of the Very Rapid Species. Spectra from ^{95}Mo-enriched samples of xanthine oxidase obtained in the present work were comparable to those in the early studies of Bray and Meriwether (1966). Figure 1 shows the effect of such enrichment upon the powder line shape of the xanthine Very Rapid EPR signal. Spectra were recorded at both X-band and Q-band microwave frequencies (Figure 1c,g). Computer simulations of this line shape (Figure 1d,h), as described under Materials and Methods, were used to determine $\mathbf{g}$ and $\mathbf{A}(^{95}\text{Mo})$ and their relative orientations. The parameters are summarized in Table I. The diagonal frames of $\mathbf{g}$ and $\mathbf{A}(^{95}\text{Mo})$ were found to be highly noncollinear, none of the axes being coincident.

2-Oxo-6-methylpurine is a convenient substrate for investigation of the Very Rapid signal because the slow turnover time permits the signal to be observed without use of rapid freezing (George, 1983; Bray & George, 1985). Figure 2 shows the effects of ^{95}Mo and ^{97}Mo on the line shape of the 2-oxo-6-methylpurine Very Rapid EPR signal. Computer simulations (Figure 2b,d,h,j) confirmed the general similarity to the xanthine Very Rapid signal. Parameters (Table I) indicate not only comparable $\mathbf{g}$ and $\mathbf{A}(^{95}\text{Mo})$ principal values but also a similar angular relationship between $\mathbf{g}$ and $\mathbf{A}$. Spectra of the 2-oxo-6-methylpurine Very Rapid signal for the ^{97}Mo-enriched enzyme (Figure 2e,k) indicate substantial effects on the lineshape due to nuclear electric quadrupole coupling. Such effects stem from the fact that the electric field gradient at the nucleus will exert a torque upon the quadrupolar nucleus. Thus, a competition arises between the magnetic hyperfine interaction and the electric field gradient for the orientation of the axis of nuclear spin. In this case M_I will not be a good quantum number, and the $2I + 1$ nuclear levels

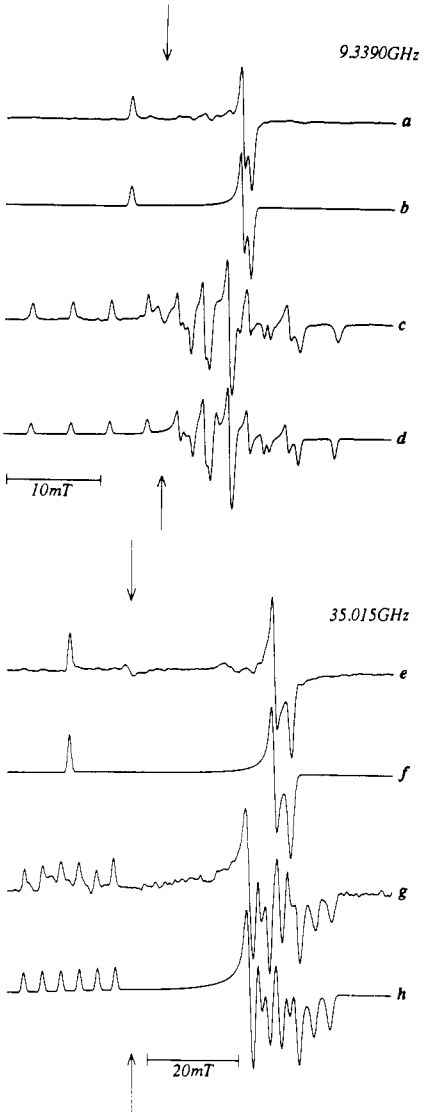

FIGURE 1: Effect of enrichment with ^{95}Mo on the xanthine Very Rapid Mo(V) EPR signal. Spectra were recorded both at X-band (a–d) and at Q-band (e–h) microwave frequencies. Computer simulations (b, d, f, and h) obtained as described in the text (see Table I for parameters) are shown below the corresponding experimental spectra (a, c, e, and g). (a) and (e) show the spectrum for unenriched enzyme and (c) and (g) that corresponding to 100% ^{95}Mo. The arrow corresponds to $g = 2.0037$. The small derivative-shaped feature near $g = 2$ in (c) and (e) is due to flavin semiquinone.

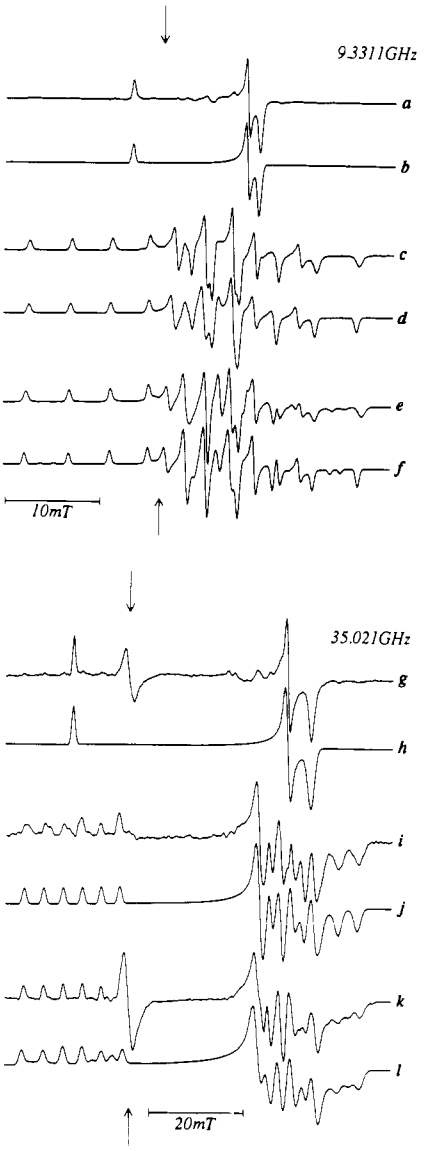

FIGURE 2: Effect of enrichment with ^{95}Mo and ^{97}Mo on the 2-oxo-6-methylpurine Very Rapid Mo(V) EPR signal. Spectra were recorded both at X-band (a–f) and at Q-band (g–l) frequencies. Computer simulations (b, d, f, h, j, and l) obtained as described in the text (see Table I for parameters) are shown below the corresponding experimental spectra (a, c, e, g, i, and k). (a) and (g) show the spectrum of unenriched enzyme, (c) and (i) those corresponding to 100% ^{95}Mo, and (e) and (k) those corresponding to 100% ^{97}Mo. The arrow corresponds to $g = 2.0037$. The derivative-shaped feature near $g = 2$ in (g) and (k) is due to flavin semiquinone.

will be unequally spaced, resulting in a rearrangement of the positions of the allowed $\Delta M_I = 0$ EPR transitions plus a marked increase in intensity of the "forbidden" $\Delta M_I = \pm 1$, $\Delta M_I = \pm 2$, $\Delta M_I = \pm 3$, $\Delta M_I = \pm 4$, and $\Delta M_I = \pm 5$ transitions. Computer simulation allows a deconvolution of the EPR powder line shape into the different ΔM_I components.

The final simulations that were achieved, on the basis of the parameters in Table I, are illustrated in Figure 2f,l. Figure 3 shows how the X-band spectrum is made up from the contributions of the different transitions. Features attributable to both allowed and "forbidden" transitions are clearly apparent in the experimental line shape (see Figures 2e and 3). The simulations (Table I) yielded a highly asymmetric quadrupole coupling $\mathbf{P}(^{97}\text{Mo})$. $\mathbf{P}(^{97}\text{Mo})$ was taken to be collinear with $\mathbf{A}(^{97}\text{Mo})$, since it was found that introducing noncollinearity between them did not produce any marked improvement in the simulation. However the largest component of **P** coincides with A_{yy} rather than with A_{zz}, and it is possible that **P** is in fact slightly rotated from **A**, although this cannot readily be established from the present data, spectra at lower microwave frequencies (S- or L-band) being required.

^{95}Mo and ^{97}Mo Isotopic Substitution of the Rapid Species. The formamide Rapid type 1 signal was selected for the present study because it has been particularly well characterized and apparently represents a single species (Morpeth et al., 1984), other Rapid signals often being mixtures [cf. Bray et al. (1978)]. The effects of enrichment with ^{95}Mo and ^{97}Mo upon the formamide Rapid EPR signal are shown in Figure 4. Simulations of the ^{95}Mo coupling (Figure 4d,j) indicate (Table I) a smaller noncollinearity between **g** and **A** than was found for the Very Rapid signal (Table I), **g** and **A** being collinear along the A_{yy} axis. Estimations of the ^{97}Mo quadrupole coupling by simulation (Figure 4f) indicated an axially symmetric **P**, which again was collinear with **A**. The line shape was found, however, to be less sensitive to the magnitude of the quadrupole interaction than was that of the Very Rapid

Table I: Parameters for the Very Rapid and Rapid EPR Signals

parameter[a]	axis[b] z	y	x	av	Euler angles[c] α	β	γ
Xanthine Very Rapid Signal							
g[d]	2.0252	1.9550	1.9494	1.9765			
A(^{95}Mo)	133.0	54.7	57.3	81.6	8	36	0
A(^{17}O)[e]	38.0	38.3	37.1	37.8			
A(^{33}S)[f]	8.5	76.6	19.1	34.7	40	0	10
2-Oxo-6-methylpurine Very Rapid Signal							
g	2.0229	1.9518	1.9446	1.9731			
A(^{95}Mo)	141.6	60.1	63.4	88.4	7	42	0
P(^{97}Mo)[g]	4.0	−5.5	1.5		7	42	0
A(^{17}O)	34.2	36.3	32.9	34.5			
A(^{33}S)	8.5	82.0	16.3	35.6	30	0	10
Formamide Rapid Type 1 Signal							
g	1.9901	1.9710	1.9666	1.9759			
A(^{95}Mo)	184.0	74.0	77.0	112.0	0	18	0
P(^{97}Mo)[g]	4.0	−2.0	−2.0		0	18	0
A(^{1}H)[h]	34.0	34.8	36.3	35.0			
	12.8	4.7	4.4	7.3			
A(^{17}O)[h]	4.2	8.3	44.0	18.8	35	0	0
A(^{33}S)[f,i]	9.5	9.9	9.9	9.8			

[a] Couplings are given in MHz; 1 mT = 27.66 MHz for $g = 1.976$; some data from the literature are included for comparison; line widths are similar to those given in earlier publications from this laboratory. Approximate errors are considered to be ±0.0003 for g values, ±1.5 MHz for hyperfine couplings, ±1.0 MHz for quadrupole couplings, and ±5% for Euler angles. [b] x, y, and z refer to the principal axes of the parameter. [c] The Euler angles in degrees are quoted corresponding to successive rotations (anticlockwise, looking toward the origin) as follows; α about z, β about the new y, and γ about the new x. All rotations are performed with reference to the principal axes of **g**. [d] Data from Tanner et al. (1978). [e] Data from Gutteridge and Bray (1980). [f] Data from Malthouse et al. (1981). [g] Because **P** is traceless, it can be represented in terms of only two parameters. Thus, quadrupole coupling constants e^2Qq and an asymmetry parameter η are sometimes quoted in the literature; these are related to **P** by $e^2Qq = 2I(2I-1)P_{zz}$ ($20P_{zz}$ for $I = {}^5/_2$) and $\eta = (P_{yy} - P_{xx})/P_{zz}$, where P_{zz} is the largest principal axis of **P**. Note that the signs given for the components of **P** are relative and not absolute. [h] Data from Morpeth et al. (1984) [note that the Euler angles for A(^{17}O) were wrongly quoted in this publication]. [i] Rapid type 1 signal generated with 1-methylxanthine.

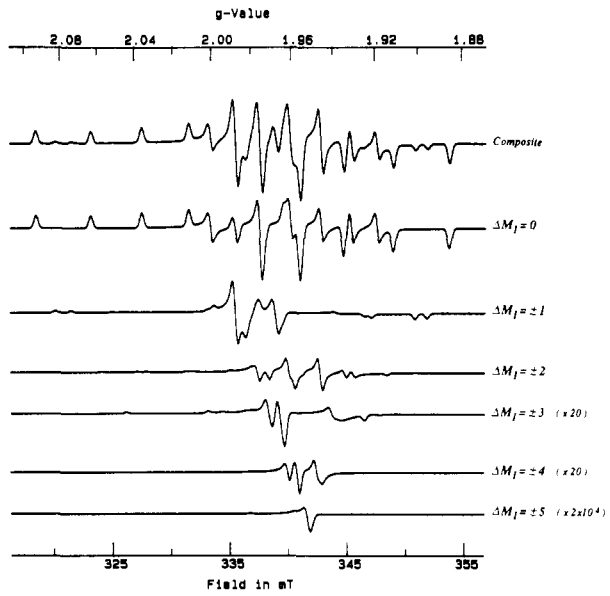

FIGURE 3: Contributions of different transitions to the line shape of the 2-oxo-6-methylpurine Very Rapid simulation (at 9.3311 GHz).

signal, and the values for **P** should be regarded as approximate.

^{17}O and ^{33}S Isotopic Substitution of the Very Rapid Species. Hyperfine couplings of Mo(V) to ^{17}O and to ^{33}S in the cyanide-labile site of the enzyme are summarized in Table I. Values of these parameters for the xanthine Very Rapid signal have been reported by Gutteridge and Bray (1980) and by Malthouse et al. (1981), respectively. We carried out analogous studies on the 2-oxo-6-methylpurine Very Rapid signal and obtained good-quality spectra (not illustrated). The ^{17}O and ^{33}S couplings (Table I) differed only slightly from those of the xanthine signal. Clearly, from the similarity of all the EPR spectral parameters (Table I), the structure at the molybdenum site of the signal-giving species is basically the same, whether the Very Rapid signal is generated with xanthine or with 2-oxo-6-methylpurine.

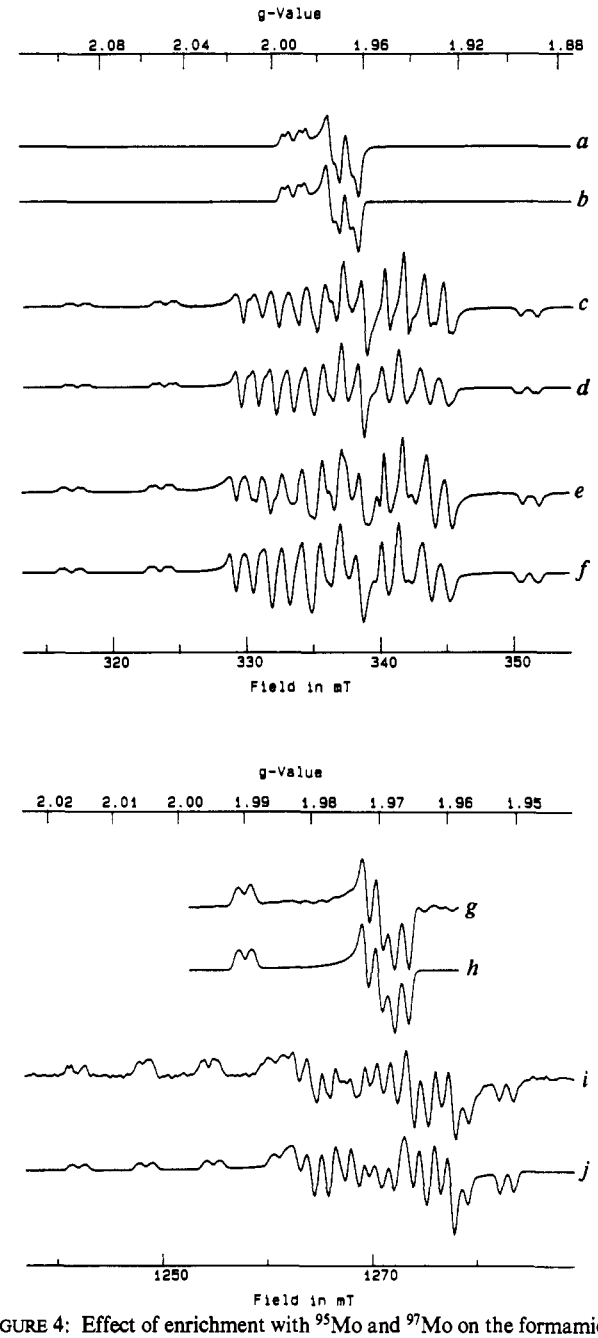

FIGURE 4: Effect of enrichment with ^{95}Mo and ^{97}Mo on the formamide Rapid type 1 Mo(V) EPR signal. Spectra were recorded both at X-band (a–f) and at Q-band (g–j) microwave frequencies (9.292 and 35.04 GHz, respectively. Computer simulations (b, d, f, h, and j) obtained as described in the text (see Table I for parameters) are shown below the corresponding experimental spectra (a, c, e, g, and i). (a) and (g) show the spectrum of unenriched enzyme [contributions from ^{95}Mo and ^{97}Mo have been subtracted from (a) to give the spectrum of 100% ^{even}Mo ($I = 0$)], (c) and (i) show those corresponding to 100% ^{95}Mo, and (e) shows that corresponding to 100% ^{97}Mo.

Discussion

Our data provide important new information bearing on the structures of Mo(V) EPR signal-giving species from xanthine oxidase. The orientation of the principal axes of **g** are determined by mixing of excited-state d orbitals into the half-occupied ground-state orbital via spin–orbit coupling. The orientations of the principal axes of the metal hyperfine interaction **A**(^{95,97}Mo), however, are expected to depend upon the basic nature of the ground state and thus are more likely to be close to the molecular (or pseudosymmetry) axes than are those of **g**. The nature of the noncollinearity between **g** and **A**(^{95,97}Mo) can give indications of the symmetry of the signal-giving species. For complexes of high symmetry, **g** and **A**(^{95,97}Mo) are required to be collinear; for those possessing only a twofold rotation axis (C_2) or a mirror plane (C_s), one principal axis will coincide, and for complexes of lower symmetry (C_1, no symmetry, or C_i, with an inversion center), none of the principal axes are required to be coincident (Hitchman et al., 1969; Belford et al., 1977; Scullane et al., 1979). From the data reported in Table I it is clear that the Rapid signal-giving species possesses metal hyperfine couplings typical of a complex of C_s or C_2 symmetry, while the hyperfine coupling of the Very Rapid species indicates that it possesses only an inversion center or, more probably, no symmetry.

Information on **g** and **A**(^{95}Mo) values of well-characterized Mo(V) compounds of potential relevance to enzyme systems has been accumulating in the literature in recent years [e.g., Scullane et al. (1979), Kaul et al. (1985), Farchione et al. (1986), Collison et al. (1986), Hanson et al. (1981, 1987), Cleland et al. (1987), Young et al. (1987), and Dowerah et al. (1987)]. However, many of the reported molybdenum hyperfine couplings have been determined [cf. Scullane et al. (1979) and Collison et al. (1986)] by simulation of the satellite lines from the 16% ^{95}Mo and 10% ^{97}Mo naturally abundant isotopes, neglecting possible quadrupole effects from the ^{97}Mo component. Because of the possible ambiguities involved, values so determined should be regarded with a certain amount of caution. In any case it is clear that for the Rapid species **g** and **A**(^{95}Mo) values are comparable to those for a number of currently available model compounds with C_s symmetry (Scullane et al., 1979; Collison et al., 1986), while, in contrast, there are no comparable models for the Very Rapid species. There is, furthermore, a dearth of information on ^{97}Mo quadrupole couplings with which to compare the data on xanthine oxidase. Nilges and Belford (1979) carried out a single-crystal EPR study on $[MoO(SCN)_5]^{2-}$ and found a nearly axial **P**(^{97}Mo) with a largest component of 2.0 MHz. Haight et al. (1979) studied a complex, presumed to be $[MoO(SCH_2CH_2S)_2]^-$, in frozen solution. In this laboratory, R. Durant, G. N. George, R. C. Bray, and G. J. Leigh (unpublished work) also investigated this complex, coonfirming **P** to be axial with a largest component of 4.0 MHz. We also studied $[MoO(SC_6H_5)_4]^-$ (Hanson et al., 1981, 1987), which again possessed an axial **P** and a largest component of 4.7 MHz.

The principal axes of the electric field gradient tensor, and thus of the quadrupole coupling tensor **P**(^{97}Mo), will be determined principally by the geometry and occupancy of the metal 4d and 5p orbitals. The populations of the various orbitals will contribute to **P** in a simple additive manner. Thus for hydrogen-like 4d orbitals, d_{xy} and $d_{x^2-y^2}$ will give a contribution proportional to $+(4/7)\langle r^{-3}\rangle_{4d}$, d_{xz} and d_{yz} to $-(2/7)\langle r^{-3}\rangle_{4d}$, and d_{z^2} to $-(4/7)\langle r^{-3}\rangle_{4d}$ [cf. Lucken (1969)]. For a Mo(V) species, therefore, we would anticipate a significant contribution to **P** from the unpaired electron in the $4d_{xy}$-type ground state, and this is expected to give a contribution to P_{zz} of about 2.9 MHz (Nilges & Belford, 1979). The nature of the bonding to the various ligands will also have large effects upon **P**; thus for an oxo group, σ and π bonding would be expected to populate those metal 5p and 4d orbitals giving negative contributions to **P**, which would result in a net decrease in P_{zz}. On the other hand, equatorial ligands would, in a similar manner, be expected to yield an increased P_{zz}. Thus the limited model compound quadrupole coupling data are simple to explain in a qualitative manner. The sulfur-ligated species $[MoO(SC_6H_5)_4]^-$ and $[MoO(SCH_2CH_2S)_2]^-$ will have large positive contributions to P_{zz} from the covalency of the equatorial sulfur ligands, while $[MoO(SCN)_5]^{2-}$ will have smaller such contributions from the nitrogen ligands. These conclusions are supported by the high g values of the sulfur-ligated species. Both types of models will have similar contribution to P_{zz} from the bonding of the oxo. For all such simple species with structures of the Mo=O^{3+} type, and similarly for Mo=S^{3+} species, we would expect **A** and **P** to be collinear, with the largest principal axis of **P** coincident with that of **A**.

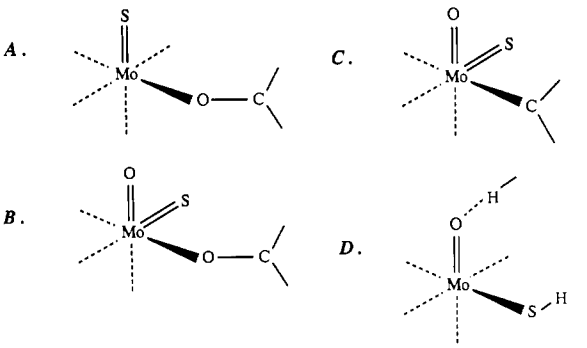

FIGURE 5: Possible structures for the Very Rapid (a–c) and Rapid (d) signal-giving species. In (a–c) the carbon is that of the substrate that is being hydroxylated. All structures are depicted with octahedral-type geometry, but it is important to note that this is not necessarily the case.

The quadrupole coupling data for the Rapid enzyme species (Table I) seems fully consistent with a Mo=O^{3+} species with a number of equatorial sulfur ligands. As discussed above the noncollinearity of the metal hyperfine coupling suggests C_s or C_2 symmetry or lower. These conclusions are consistent with the structure proposed by Morpeth et al. (1984) from the ligand hyperfine couplings, which is shown in Figure 5D. Confidence that the Rapid signal-giving species possess a structure similar to Figure 5d is strengthened by recent model compound work (Farchione et al., 1986; Dowerah et al., 1987).

The case of the Very Rapid species is clearly not as simple. As discussed above, **P** is highly anisotropic, with the largest principal axis pointing along A_{yy}; this seems inconsistent with a simple Mo=O^{3+} or Mo=S^{3+} site. On the other hand, a molybdenum site with both oxo and sulfido ligands, for which no fully authenticated Mo(V) model compounds have been reported, would have large contributions to **P** from the σ and π bonding of the terminal sulfur, and although prediction of the exact nature of **P** is beyond the scope of the present work, it seems clear that one might expect a highly asymmetric **P** for such a site. Thus, the quadrupole coupling data appear most consistent with an oxo–sulfido-type structure for the Very Rapid signal-giving species. In the light of the new data we will now discuss further the structure of the Very Rapid species.

Several possible structures that have been or can be suggested for the Very Rapid Species are illustrated in Figure 5A–C. All of these are compatible with the proposed role

(Bray & George, 1985; Bray, 1987) of the Very Rapid species [or its Mo(IV) analogue] as an intermediate of catalytic turnover. The structure first proposed (Gutteridge & Bray, 1980; Bray & Gutteridge, 1982) for the xanthine Very Rapid species is that of Figure 5A and was based on the following information. Experiments with xanthine enriched with ^{13}C in the C-8 position (the position that is hydroxylated by the enzyme) indicated a weak hyperfine coupling to the ^{13}C nucleus in the Very Rapid species with $A(^{13}C)_{av}$ = 8.9 MHz (Tanner et al., 1978), suggesting that the xanthine is covalently associated with molybdenum in the signal-giving species. Comparable results have since been obtained with ^{13}C-enriched 1-methylxanthine (S. Gutteridge, R. C. Bray, and F. Bergmann, unpublished work). Work with ^{17}O-enriched enzyme (Gutteridge & Bray, 1980) indicated an apparently isotropic coupling to oxygen, from which Gutteridge and Bray concluded that the xanthine was probably bound via a Mo–O–C linkage. ^{33}S-enriched enzyme gave a highly anisotropic hyperfine coupling to ^{33}S (Malthouse & Bray, 1980; Malthouse et al., 1981) which, together with the absence of hyperfine coupling to exchangeable protons [cf. George (1985)], was taken to be consistent with a Mo=S species as in Figure 5A.

The size of the coupling to ^{33}S (Malthouse et al., 1981; George, 1983) is certainly a unique spectral feature of the Very Rapid signal, in comparison with other known Mo(V) species. ^{33}S possesses a small nuclear g value, and hyperfine couplings to it tend to be correspondingly small. Thus, an unpaired electron residing entirely in a sulfur 3p orbital would be expected to give an anisotropic coupling of only 156 MHz (Goodman & Raynor, 1970). The anisotropy of the measured coupling to ^{33}S in the Very Rapid signal (Table I) indicates a minimum anisotropic component of $\mathbf{A}(^{33}S)$ close to 60 MHz [cf. George (1983)], some 38% of the calculated value for a unit population on sulfur. This clearly indicates that the cyanolysable sulfur participates to a large extent in the ground-state molecular orbital of the signal-giving species. Thus, it seems probable that the unusually large value for g_{zz}, which has long been taken as evidence for sulfur ligation, results mainly from spin–orbit coupling with filled orbitals involving this sulfur.

Because of the above considerations, and as initially pointed out by Bray and George (1985), the structure of Figure 5A is obviously incomplete. If the unpaired electron is extensively delocalized on to the sulfido group, then another group, exerting a stronger ligand field, is required to bring the ground-state orbital into approximately the same plane as the Mo=S group. Bray and George (1985) postulated an additional oxo group, cis to the sulfido, as in Figure 5B to fulfill this requirement. In support of these arguments, Young et al. (1987) have recently reported the optical and EPR spectroscopic properties of a mononuclear Mo^{V}=S compound, which should serve as a model for the structure of Figure 5A. Interestingly, the compound has slightly lower g values than its Mo^{V}=O analogue. Young et al. (1987) explain this in terms of the smaller ligand field splitting expected from a sulfido, relative to an oxo. This finding contrasts with the data from the Very Rapid species, which, as already stated, has an large unusually g_{zz} (Table I). Also, although Young et al. (1987) did not perform isotopic enrichment experiments, a large coupling to ^{33}S would clearly not be expected from the axially located terminal sulfur atom of their compound.

The EPR spectra of several mononuclear Mo(V) species of significance to molybdoenzymes have recently been reported in an elegant study by Dowerah et al. (1987). All the complexes studied by these workers were solution species obtained by chemical modification of the Mo(VI) dioxo species $LMoO_2$ [where LH_2 is N,N'-dimethyl-N,N'-bis(2-mercaptophenyl)-ethylenediamine]. The complexes obtained were attributed to $[LMoO_2]^-$, $[LMoOS]^-$, LMoO(OH), and LMoO(SH) species. The EPR spectra of LMoO(OH) and LMoO(SH) showed proton hyperfine coupling and are analogous to the xanthine oxidase Mo(V) Slow and Rapid species, respectively. Of particular relevance to the Very Rapid is the species generated by reduction of $LMoO_2$ with SH^-, which Dowerah et al. (1987) attribute to the Mo(V) complex $[LMoOS]^-$, which presumably has oxo and sulfido ligands. This species has EPR parameters that are distinct from those of the Very Rapid species, possessing a highly anisotropic **g** tensor (1.8885, 1.9330, 2.0160). From computer simulations of the natural abundance Mo X-band data, Dowerah et al. (1987) also report that **g** and $\mathbf{A}(^{95,97}Mo)$ are collinear (which is unexpected on symmetry grounds), with $\mathbf{A}(x,y,z)$ = 116, 71, and 141 MHz. If the complex of these workers is indeed an oxo–sulfido species, then the EPR spectral parameters, particularly the reported collinearity of **g** and **A**, might be said to argue against such a structure (Figure 5B) for the Very Rapid species. However, unambiguous interpretation of data on the model compound is scarcely possible. In contrast to the situation for the Very Rapid signal, which has a nearly axial **g**, the putative $[LMoOS]^-$ has a highly rhombic **g**. In such circumstances, unless low microwave frequencies (e.g., 3 GHz) are used, noncollinearity of **g** and **A** will not normally produce observable distortions of the powder line shape from that expected for collinear **g** and **A**; this is particularly so if unenriched samples are used. Thus we found that essentially indistinguishable X-band simulations were obtained with the parameters of Dowerah et al. (1987) or with unchanged **g** and $\mathbf{A}(x,y,z)$ = 71, 71, and 158 MHz, with $\beta = 35°$. It seems therefore that the parameters of the model compound, its relation to the Very Rapid species, and its structure have yet to be firmly established.

Although difficult to rationalize mechanistically, a further structure, shown in Figure 5C, for the Very Rapid species can be considered, in which the substrate carbon is coordinated directly to the molybdenum. This structure is fully consistent with the ^{97}Mo, ^{95}Mo, and ^{33}S data discussed above. The direct molybdenum–carbon bond is, furthermore, consistent with the data on ^{13}C coupling for the Very Rapid signal (Tanner et al., 1978; S. Gutteridge, R. C. Bray, and F. Bergmann, unpublished work). The coupling to ^{13}C is small (8.9 MHz for xanthine), and this is expected in d^1 systems for equatorial ligands σ bonded to the metal, because the ligand lies on a node of the ground-state orbital.

Even if indirect evidence points toward an oxo group in the Very Rapid species, definitive evidence is at present lacking. Such evidence might come from EXAFS spectroscopy. Some preliminary EXAFS data (George et al., 1986) relating to the Very Rapid species are available and do appear to support the presence of an oxo group; however, these results should be treated with caution until more detailed kinetic work [cf. Davis et al. (1984)] is done. On the other hand, whether such a structure is compatible with the ^{17}O data (Table I) is debatable. The ^{17}O coupling of the axial oxo of $[MoO(SC_6H_5)_4]^-$ has been determined by Hanson et al. (1987) to be small and quite anisotropic $[\mathbf{A}(^{17}O)(x,y,z)$ = 8.6, 8.6, and 1.9 MHz]. Indeed, the main evidence for the presence of a Mo–O– linkage in the signal-giving species as in Figure 5A,B is that the ^{17}O hyperfine coupling (Table I) is observed to be largely isotropic. However, nothing is known about the signs of the ^{17}O coupling, and it cannot be excluded that one of the principal components

is of diferent sign to the other two, giving the appearance of a very small anisotropic component in the powder line shape. Thus, neglecting the effects of any noncollinearity, an anisotropic coupling of +48 MHz with an isotropic coupling of −12 MHz (the signs are relative, not absolute) would be an alternative interpretation of the ^{17}O data. This can be compared with the measured anisotropic ^{17}O hyperfine coupling of the Rapid signal (Morpeth et al., 1984; see Table I) and the Mercurial signal (George & Bray, 1983), which has a largest principal component of 34 MHz. It should also be noted that the coupling to ^{17}O in an oxo–sulfido site, as postulated in Figure 5C, might be expected to be very different from the simple MoO^{3+} compound investigated by Hanson et al. The possibility that the oxygen hyperfine coupling of the Very Rapid signal arises from a terminal oxo group in the signal-giving species, as in Figure 5C, rather than a Mo–O–C group as in Figure 5B, thus cannot be excluded.

ACKNOWLEDGMENTS

We thank Dr. P. Young and Dr. M. J. Ducker of the National Institute for Research in Dairying for providing buttermilk from cows injected with Mo isotopes, Ruth Williams for assistance, and Prof. R. L. Belford for making the program QPOW available to us. Some of the work of G.N.G. was under a studentship from the SERC.

REFERENCES

Anderson, R. H., Hille, R., & Massey, V. (1986) *J. Biol. Chem. 261*, 15870–15876.

Belford, R. L., Harrowfield, B., & Pilbrow, J. R. (1977) *J. Magn. Reson. 28*, 433–439.

Blumer, D. J., Cheng, C. P., & Brown, T. L. (1977) *Chem. Phys. Lett. 51*, 473–476.

Bordas, J., Bray, R. C., Garner, C. D., Gutteridge, S., & Hasnain, S. S. (1980) *Biochem. J. 191*, 499–508.

Bray, R. C. (1975) *Enzymes (3rd Ed.) 12*, 299–419.

Bray, R. C. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol. 51*, 107–165.

Bray, R. C. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 775–785, Elsevier, Amsterdam.

Bray, R. C. (1988) *Q. Rev. Biophys.* (in press).

Bray, R. C., & Meriwether, L. S. (1966) *Nature (London) 212*, 467–469.

Bray, R. C., & Gutteridge, S. (1982) *Biochemistry 21*, 5992–5999.

Bray, R. C., & George, G. N. (1985) *Biochem. Soc. Trans. 13*, 560–567.

Bray, R. C., Barber, M. J., & Lowe, D. J. (1978) *Biochem. J. 171*, 653–658.

Cleland, W. E., Barnhart, K. M., Yamanouchi, K., Collison, D., Mabbs, F. E., Ortega, R. B., & Enemark, J. H. (1987) *Inorg. Chem. 26*, 1017–1025.

Collison, D., Mabbs, F. E., Enemark, J. H., & Cleland, W. E. (1986) *Polyhedron 5*, 423–425.

Cramer, S. P., & Hille, R. (1985) *J. Am. Chem. Soc. 107*, 1864–1869.

Cramer, S. P., Wahl, R., & Rajagopalen, K. V. (1981) *J. Am. Chem. Soc. 103*, 7721–7727.

Davis, M. D., Olson, J. S., & Palmer, G. (1984) *J. Biol. Chem. 259*, 3526–3533.

Dowerah, D., Spence, J. T., Singh, R., Wedd, A. G., Wilson, G. L., Farchione, F., Enemark, J. H., Kristofzski, J., & Bruck, M. (1987) *J. Am. Chem. Soc. 109*, 5655–5665.

Farchione, F., Hanson, G. R., Rodrigues, C. G., Bailey, T. D., Bagchi, R. N., Bond, A. M., Pilbrow, J. R., & Wedd, A. G. (1986) *J. Am. Chem. Soc. 108*, 831–832.

George, G. N. (1983) D.Phil. Thesis, University of Sussex.

George, G. N. (1985) *J. Magn. Reson. 64*, 384–394.

George, G. N., & Bray, R. C. (1983) *Biochemistry 22*, 5443–5452.

George, G. N., Bray, R. C., & Cramer, S. P. (1986) *Biochem. Soc. Trans. 14*, 651–652.

Goodman, B. A., & Raynor, J. B. (1970) *Adv. Inorg. Chem. Radiochem. 13*, 135–362.

Gutteridge, S., & Bray, R. C. (1980) *Biochem. J. 189*, 615–623.

Gutteridge, S., Tanner, S. J., & Bray, R. C. (1978) *Biochem. J. 178*, 887–897.

Haight, G. P., Belford, R. L., & Chapman, H. (1979) in *Chemistry and Uses of Molybdenum* (Barry, H. F., & Mitchell, P. C. H., Eds.) pp 245–248, Climax Molybdenum Co., Ann Arbor, MI.

Hanson, G. R., Brunette, A. A., McDonnel, A. C., Murray, I. T. S., & Wedd, A. G. (1981) *J. Am. Chem. Soc. 103*, 1953–1959.

Hanson, G. R., Wilson, G. L., Bailey, T. D., Pilbrow, J. R., & Wedd, A. G. (1987) *J. Am. Chem. Soc. 109*, 2609–2616.

Hart, L. I., McGartoll, M. A., Chapman, H. R., & Bray, R. C. (1970) *Biochem. J. 116*, 851–864.

Hille, R., & Massey, V. (1985) in *Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 443–518, Wiley, New York.

Hitchman, M. A., Olson, C. D., & Belford, R. L. (1969) *J. Chem. Phys. 50*, 1195–1203.

Kaul, B. B., Enemark, J. H., Merbs, S. L., & Spence, J. T. (1985) *J. Am. Chem. Soc. 107*, 2885–2891.

Lucken, E. A. C. (1969) in *Nuclear Quadrupole Coupling Constants*, Academic, New York.

Malthouse, J. P. G., & Bray, R. C. (1980) *Biochem. J. 191*, 265–267.

Malthouse, J. P. G., George, G. N., Lowe, D. J., & Bray, R. C. (1981) *Biochem. J. 197*, 421–425.

Morpeth, F. F., George, G. N., & Bray, R. C. (1984) *Biochem. J. 220*, 235–242.

Nilges, M. J., & Belford, R. L. (1979) *J. Magn. Reson. 35*, 259–281.

Scullane, M. I., Taylor, R. D., Minelli, M., Spence, J. T., Yamanouchi, I. T., Enemark, J. H., & Chasteen, N. D. (1979) *Inorg. Chem. 18*, 3214–3219.

Seebauer, E. G., Duliba, E. P., Scogin, D. A., Gennis, R. A., & Belford, R. L. (1983) *J. Am. Chem. Soc. 105*, 4926–4929.

Stiefel, E. I. (1977) *Prog. Inorg. Chem. 22*, 1–223.

Stiefel, E. I., Newton, W. E., & Pariyadath, N. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H., & Seaman, A., Eds.) pp 265–270, Climax Molybdenum Co., London.

Tanner, S. J., Bray, R. C., & Bergmann, F. (1978) *Biochem. Soc. Trans. 6*, 1327–1330.

Young, C. G., Collison, D., Mabbs, F. E., & Enemark, J. H. (1987) *Inorg. Chem. 26*, 2925–2927.