Studies by electron-paramagnetic-resonance spectroscopy of the environment of the metal in the molybdenum cofactor of molybdenum-containing enzymes

Timothy R. HAWKES* and Robert C. BRAY

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

(Received 14 February 1984/Accepted 29 May 1984)

The molybdenum cofactor prepared by denaturing xanthine oxidase by heat treatment or other methods was partially purified by anaerobic gel filtration in the presence of sodium dithionite, with little loss of activity. A range of products with different elution volumes was obtained. This behaviour is apparently related to association of the molybdenum cofactor with various residual peptides. E.p.r. signals from molybdenum(V) in the active cofactor, present either in crude preparations or in purified fractions, may be generated in dimethyl sulphoxide solution by controlled oxidation carried out on the molybdenum cofactor alone or in the presence of added thiols. The g -values of the spectra suggest that in the oxidized cofactor molybdenum has one terminal oxygen ligand and four ligands from thiolate groups. It is proposed that two of these are from the organic part of the cofactor and two from cysteine residues in the protein or in residual peptides. A signal generated in high yield with little loss of cofactor activity in the presence of thiophenol has $g_{\parallel} = 2.0258$ and $g_{\perp} = 1.9793$. It is suggested that in this species two cysteine residues have been replaced by two thiophenol molecules. The possible usefulness of the thiophenol complex in further purification of the molybdenum cofactor is discussed.

The molybdenum cofactor (Johnson, 1980) is a constituent of all molybdenum-containing enzymes other than nitrogenase. Its existence was first proposed by Pateman *et al.* (1964). However, progress in characterizing it has been slow. This is largely because of its instability in air (see, e.g., Lee *et al.*, 1974). The cofactor is generally assayed (Johnson, 1980) by its ability to complement the apo nitrate reductase, present in extracts of the *nit-1* mutant of *Neurospora crassa*, to yield active nitrate reductase. Nevertheless, it is only recently that conditions have been described in detail (Hawkes & Bray, 1984) for handling the molybdenum cofactor without loss of activity and for assaying it quantitatively on an absolute basis. An inactive oxidized degradation product, which does not contain the metal, has been partly characterized and shown (Johnson *et al.*, 1980; Johnson & Rajagopalan, 1982) to contain a pterin derivative related to urathione. Limited work only has been reported on the partial purification of the active cofactor (Johnson *et al.*, 1980; Alikulov *et al.*,

1980; Claassen *et al.*, 1982; Cramer & Minak, 1983), and molybdenum has often been lost from it (see, e.g., Alikulov *et al.*, 1980) during purification.

None of the molybdenum-containing enzymes has yet been characterized by X-ray crystallography. However, e.p.r. spectroscopy of their molybdenum in the Mo(V) oxidation state has yielded a wealth of information bearing on the structures and mechanisms of action of their molybdenum centres (Bray, 1980a,b; Bray *et al.*, 1983). Despite the obvious potentiality of e.p.r. for probing the ligation of molybdenum in the cofactor, no detailed e.p.r. studies have yet been reported on it. In the only two attempts in this direction, L'vov *et al.* (1975) presented ill-defined e.p.r. spectra, and Johnson (1980), though presenting a well-defined spectrum, did not give accurate g -values or indicate the fractional conversion of the metal into the Mo(V). More importantly, she did not provide evidence that the spectrum was from active molybdenum cofactor rather than from some degradation product.

We now report partial purification of the molybdenum cofactor, carried out in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ to keep the cofactor fully reduced and so prevent (Hawkes & Bray, 1984) dissociation of the

* Present address: Agricultural and Food Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton BN1 9RQ, U.K.

metal. We have carried out a detailed investigation of a number of e.p.r.-active species from the active molybdenum cofactor, permitting us to draw tentative conclusions concerning the ligation of molybdenum.

Materials and methods

Materials

Dimethyl sulphoxide was of spectroscopic grade from Fluka (via Fluorochem, Glossop, Derbyshire, U.K.); *N*-methylformamide (from Aldrich Chemical Co., Gillingham, Dorset, U.K.) was dried over Na_2CO_3 , redistilled under vacuum and stored under N_2 . Chemicals and biochemicals were generally the purest grade available from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma Chemical Co. (Poole, Dorset, U.K.). Sephadex LH-20 and G-25 (fine grade) were from Pharmacia (Hounslow, Middx., U.K.). $\text{NHET}_3\text{-[MoO(SC}_6\text{H}_5)_4]$, prepared as described by Boyd *et al.* (1978), was provided by Dr. G. N. George; the ^{95}Mo -enriched compound, similarly prepared, was provided by Dr. R. Durrant. Mediator oxidation-reduction dyes were as follows: Phenosafranine and Indigo Carmine (BDH Chemicals), Safranin-T (Fluka), 2-hydroxy-1,4-naphthoquinone (Koch-Light Laboratories, Colnbrook, Bucks., U.K.), anthroquinone-1,5-disulphonate (Aldrich Chemical Co.), gallocyanine (Hopkin and Williams, Chadwell Heath, Essex, U.K.) and 2,5-dihydroxy-*p*-benzoquinone (Eastman Kodak, Rochester, NY, U.S.A.).

Molybdoenzymes

Xanthine oxidase was prepared and assayed as described previously (Hart *et al.*, 1970; Bray, 1975, 1982). About 70% of the enzyme in the samples used was present in the functional form. Conversion into the desulpho form was carried out as described by Massey & Edmondson (1970). ^{95}Mo -enriched xanthine oxidase, prepared as described by Bray & Meriwether (1966), was a sample stored in liquid N_2 since 1968. Sulphite oxidase was prepared as described by Lamy *et al.* (1980).

Analyses for molybdenum and for protein

Molybdenum analysis was carried out colorimetrically, after digestion with HClO_4 and H_2SO_4 (Hart *et al.*, 1970). When dimethyl sulphoxide was present, it was first removed by evaporation at 40 Pa (0.3 mmHg) and 30–40°C. Protein was determined (Sedmak & Grossberg, 1977) by using Coomassie Blue (Coomassie Brilliant Blue G-250 from Serva, Heidelberg, Germany) with bovine serum albumin as a standard; dimethyl sulphoxide, when present, was removed as described above.

Preparation and partial purification of the active molybdenum cofactor

Procedures were generally as described by Hawkes & Bray (1984); solutions of the molybdenum cofactor were always prepared and maintained in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, under anaerobic conditions. Unless otherwise stated, these solutions were prepared immediately before use, by adding 1 vol. of active xanthine oxidase to 18–40 vol. of dimethyl sulphoxide, giving the following final concentrations: 3–7% (v/v) water, 1–3 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and 5–15 mM-potassium phosphate buffer (pH 7.8 in water). After centrifugation to remove precipitated protein, cofactor concentrations corresponded to 10–18 μM -Mo, and the solutions contained 0.4–1.5 mg of residual protein/ml. In some experiments, dimethyl sulphoxide was replaced by *N*-methylformamide; other methods of extracting the molybdenum cofactor were as described by Hawkes & Bray (1984). Freshly prepared cofactor solutions had activity (see below) of about 22–24 μmol of NO_2^-/min per ng-atom of Mo, as expected for maximum activity.

Gel-filtration experiments were carried out in glass chromatography columns connected with stainless-steel tubing. Fractions were collected by hand, in tubes flushed with purified N_2 . When it was necessary to concentrate aqueous molybdenum cofactor solutions containing small amounts of dimethyl sulphoxide, this was achieved by evaporating the water in a closed system under high vacuum at 20–25°C, while the solution was stirred, until a concentration of about 90% dimethyl sulphoxide was attained.

Inactivated molybdenum factor

Solutions of the cofactor (prepared from active xanthine oxidase in dimethyl sulphoxide, as described above) were exposed to air at 20–25°C for 2 days, resulting in complete loss of activity (Hawkes & Bray, 1984).

Assay of molybdenum cofactor activity

This was carried out as described by Hawkes & Bray (1984), by aerobic complementation for 24 h at 3.5°C, of the apo nitrate reductase of partially purified extracts of the *nit-1* mutant of *Neurospora crassa*, to yield active nitrate reductase. To obtain information about the extent to which molybdenum had dissociated from otherwise intact cofactor molecules (Hawkes & Bray, 1984; see caption to Table 3, below), assays were carried out with and without addition of Na_2MoO_4 (10 mM) to the complementation medium. To decrease the quantities of *nit-1* extracts required, molybdenum cofactor samples were usually diluted 10-fold

(anaerobically with dimethyl sulphoxide containing $\text{Na}_2\text{S}_2\text{O}_4$ and potassium phosphate buffer, pH 7.8 in water) before addition to the complementation medium.

Controlled oxidations in dimethyl sulphoxide for generating e.p.r. signals

Oxidations were carried out under purified N_2 in a potentiometric titration cell fitted with platinum and calomel electrodes (Cammack *et al.*, 1976). Reaction conditions were monitored by measuring the potentials between the electrodes (generally under non-equilibrium conditions), with a millivoltmeter (input impedance 1 G Ω), with correction to the hydrogen-electrode scale by subtracting 241 mV. Samples, in dimethyl sulphoxide containing $\text{Na}_2\text{S}_2\text{O}_4$, were placed in the titration cell, with or without addition of thiols (see below). In different experiments, active molybdenum cofactor or the inactivated form were used, or these were replaced by Na_2MoO_4 . Redox mediator dyes, as indicated above (final concentration 40 μM of each), were then added; in all cases the initial potential was approx. -650 mV . The potential was then raised to the required value by addition of aqueous $\text{K}_3[\text{Fe}(\text{CN})_6]$, and maintained within the required range for an appropriate period (at $20\text{--}25^\circ\text{C}$). Further additions of $\text{K}_3[\text{Fe}(\text{CN})_6]$ were sometimes made, if the potential tended to drift. (Downward drifting was particularly apparent when thiols were present.) When it was desired to lower the redox potential, this was achieved by the addition of aqueous $\text{Na}_2\text{S}_2\text{O}_4$. The final water content of the dimethyl sulphoxide solution was usually below 8% (v/v). Samples of the solution were quickly removed, as required, anaerobically by means of syringes, for freezing in e.p.r. tubes or for cofactor assays.

Appropriate conditions for generating the various signals from the molybdenum cofactor were as follows. For signal 1, the potential was raised to $-60 \pm 50\text{ mV}$ and samples were withdrawn from the titration cell and frozen 1–2 min later. For signal 2, the potential was raised, initially to $-40 \pm 30\text{ mV}$, and the cofactor was then left for 10–60 min, without further additions, before samples were withdrawn, by which time the potential had usually fallen to about -150 mV . For signal 1_{ip}, 0.1 M-thiophenol was added, and the potential was rapidly raised to $-50 \pm 45\text{ mV}$ and samples were withdrawn and frozen within 30–45 s. (For experiments in e.p.r. tubes, this potential could be adequately judged from the colour of the mediators.) Slightly larger 1_{ip} signals were obtained either if rapid downward drifting of the potential was checked by further additions of $\text{K}_3[\text{Fe}(\text{CN})_6]$, to maintain the potential for 1–2 min before removal of the samples, or alternatively, if the

sample was re-reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and then re-oxidized for a second time, before freezing. Solutions sometimes became turbid after oxidation (particularly when thiophenol was present), but this caused no obvious problems.

E.p.r. measurements

Spectra were recorded on a Varian E9 spectrometer linked to a computer and visual-display system (Bray *et al.*, 1978). A dual-sample cavity was employed, containing manganese and diphenylpicrylhydrazyl standards (Swartz *et al.*, 1972). *g*-Value measurements are believed to be accurate to ± 0.0003 . Integrations, for absolute quantification of the signals, were carried out by using Cu^{2+} -EDTA as standard, with corrections for transition probability (Aasa & Vänngård, 1975) and for molybdenum hyperfine lines not included in the integrations, as well as for expansion of water (but not of dimethyl sulphoxide) on freezing. Conversions of the molybdenum cofactor into the Mo(V) state are expressed as corrected signal intensities, divided by the molybdenum concentration, determined colorimetrically (see above).

When it was necessary to thaw samples in e.p.r. tubes after recording e.p.r. spectra, this was carried out with repeated alternate evacuation and flushing with purified N_2 before and during the thawing process.

Results

Partial purification of the molybdenum cofactor

Despite its instability towards O_2 , Hawkes & Bray (1984) found that the molybdenum cofactor, when kept fully reduced in the presence of dithionite, was quite stable in solution in either water or dimethyl sulphoxide. These workers described conditions under which the cofactor could be liberated from molybdoenzymes. In principle, purification of the molybdenum cofactor, liberated for example by denaturation of purified xanthine oxidase with dimethyl sulphoxide, ought to be a simple task, under appropriate anaerobic conditions, since it should be contaminated only with denatured protein, FAD and Fe.

The work described below shows clearly, however, that, under good anaerobic conditions, separation of the active molybdenum cofactor from protein is not simple. In one experiment molybdenum cofactor solution was prepared by anaerobic addition of xanthine oxidase to dimethyl sulphoxide (see the Materials and methods section). About three-quarters of the protein originally present in the xanthine oxidase was removed by centrifugation, with the rest of the protein remaining in solution in the organic solvent. The

sample (1.5 ml) was applied to a column (1.0 cm $\times$ 36 cm) of Sephadex LH20, and eluted with dimethyl sulphoxide containing 3% (v/v) water, 6 mM-potassium phosphate buffer (pH 8.0 in water), and approx. 2 mM- $\text{Na}_2\text{S}_2\text{O}_4$. The residual protein emerged in the excluded fraction (7–13 ml), which also contained 85% of the cofactor activity. However, whereas the protein peak was sharp, that for the cofactor activity was less sharp, with a significant tail of activity extending up to elution volumes of 40 ml or more. Recovery of cofactor activity applied to the column (about 600 μmol of NO_2^-/min) was quantitative (106%). The activity of the fractions from the column was decreased by only about 25% if molybdate was omitted from the assay (see the Materials and methods section), thus indicating (cf. Table 3 caption) no significant loss of molybdenum from the cofactor during purification.

In further related experiments, we liberated the molybdenum cofactor from xanthine oxidase, either by using sodium dodecyl sulphate or by thermal denaturation, and carried out subsequent gel filtration in aqueous solution, rather than in dimethyl sulphoxide. Comparable results were obtained by either method; only that employing thermal denaturation is described in detail in the present paper (Table 1). In thermal denaturation, only about one-third of the cofactor is liberated into solution (Hawkes & Bray, 1984); also, in filtering the cofactor solution (Table 1, Expt. 1) about half of it was lost mechanically. Gel filtration, on Sephadex G-25, was then carried out, with 83% re-

covery of activity. As in gel filtration in dimethyl sulphoxide, activity emerged mainly along with residual protein, with a tail of activity, that was protein-free by the Coomassie Blue test, extending to high elution volumes. Again, the activity was only slightly diminished when molybdate was omitted from the assay, confirming that molybdenum had not dissociated from the cofactor.

The most obvious explanation of the behaviour observed in the experiments described above is that none of the methods of protein denaturation liberates the molybdenum cofactor completely from the xanthine oxidase protein, even though the association is sufficiently loosened to permit the cofactor to be transferred quantitatively (Hawkes & Bray, 1984) to the apo nitrate reductase in the cofactor assay. This would explain the bulk of the molybdenum cofactor emerging from the column along with the protein fraction. The tail of cofactor activity either would then represent a small proportion of molybdenum cofactor molecules that had dissociated fully from the protein and been retarded on the column, or alternatively it could represent molybdenum cofactor associated, not with the intact protein, but with relatively small peptide molecules derived from it. This latter possibility is made plausible by the facts, firstly, that the xanthine oxidase preparation (Hart *et al.*, 1970) involves extensive treatment with proteolytic enzymes, and, secondly, that the method of protein detection used (Sedmak & Grossberg, 1977) does not respond to peptides of M_r less than about 3000.

Finally, for e.p.r. spectroscopy (see below), we

Table 1. *Partial purification by gel filtration in aqueous solution, and concentration, of the molybdenum cofactor obtained by thermal denaturation of xanthine oxidase*

Thermal denaturation was carried out anaerobically as described by Hawkes & Bray (1984), and the supernatant solution was passed through a 0.8 μm -pore-size filter. Quantities of xanthine oxidase used in Expts. 1 and 2 were 25 mg and 50 mg respectively. Gel filtration, on a column of Sephadex G-25 (1.5 cm $\times$ 14 cm), was carried out anaerobically, at 4°C, in 20 mM-potassium phosphate buffer, pH 7.4, containing 2 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and 15% (v/v) methanol for Expt. 1, and, for Expt. 2, in 10 mM-potassium phosphate buffer, pH 7.8, containing 1 mM- $\text{Na}_2\text{S}_2\text{O}_4$, with no methanol. In Expt. 2, 2 ml of dimethyl sulphoxide was added to the protein-free fraction from the column, and this was then concentrated by evaporation (see the Materials and methods section). Protein was determined with Coomassie Blue, and cofactor activity was measured (with addition of Na_2MoO_4), as described in the Materials and methods section.

Expt. no.	Sample	Volume (ml)	Total cofactor activity (μmol of NO_2^-/min)	Total protein (mg)
1	Filtered supernatant from thermal denaturation of xanthine oxidase	2.4	560	0.45
	After Sephadex G-25 (protein-containing fraction)	19	380	0.35
	After Sephadex G-25 (protein-free fraction)	24	85*	Not detectable
2	After Sephadex G-25 (protein-free fraction)	10	500	< 0.05
	After addition of dimethyl sulphoxide and evaporation	2.1†	400	< 0.05

* Cofactor activity, measured without addition of Na_2MoO_4 , was 69 μmol of NO_2^-/min .

† The Mo content of the sample, determined colorimetrically, was 21 nmol.

Fig. 1. E.p.r. spectra of the molybdenum cofactor generated alone or in the presence of thiophenol

Signals were generated and recorded as described in the Materials and methods section. (a) shows signal 1 generated under the usual conditions with unpurified molybdenum cofactor in dimethyl sulphoxide and in the presence of potassium phosphate buffer. (b) shows signal 1 obtained in the same medium but with protein-free molybdenum cofactor (see Table 1, Expt. 2). (c) shows signal 1 generated with *N*-methylformamide as solvent in the presence of Tris/HCl buffer (see Table 2). (d) shows signal 2 obtained as for signal 1 in (a), but with the incubation with $K_3[Fe(CN)_6]$ prolonged for 30 min. A small amount of signal 1 is also present in this spectrum. (e) shows signal 2, fully developed and without signal 1, and was obtained by extending the incubation to 90 min. (f) shows the spectrum of a sample of inactivated molybdenum cofactor, treated under the conditions for generating signal 1, as in (a). (g) shows signal 1_{tp}; conditions for generating it were exactly as for (a) except that 0.1 M-thiophenol was

concentrated the protein-free fraction (Table 1, Expt. 2) from a Sephadex G-25 column and transferred it to dimethyl sulphoxide solution. The product had a cofactor activity of $19 \mu\text{mol}$ of NO_2^-/min per ng-atom of Mo, based on the measured Mo content of the final solution. This corresponds to about 80% of the activity (Hawkes & Bray, 1984) of fresh undegraded cofactor. We compared stability towards inactivation by air of protein-free molybdenum cofactor with that reported by Hawkes & Bray (1984) for the unfractionated cofactor. Stability, both in the absence and in the presence of dithiothreitol, was similar to that reported in the previous work.

Mo(V) e.p.r. signals from the molybdenum cofactor alone, or in the presence of thiophenol

Most of our e.p.r. work was carried out on unpurified molybdenum cofactor obtained by treatment of active xanthine oxidase with dimethyl sulphoxide in the presence of $\text{Na}_2\text{S}_2\text{O}_4$ (Hawkes & Bray, 1984; see the Materials and methods section). We obtained, under different conditions, two distinct molybdenum(V) e.p.r. signals from the cofactor, which are referred to in the present paper as signals 1 and 2. [Note that signal 1 is quite similar to a signal, possibly from the molybdenum cofactor, presented by Johnson (1980).] The addition of various thiols to the medium led to the appearance of further signals; these are indicated by the number of the original signal, followed by the initials of the thiol (thiophenol = tp; mercaptoethanol = me; ethanedithiol = ed), thus, e.g., signal 1_{tp}.

We consider, first, the signals obtained from the molybdenum cofactor on its own; these were generated by controlled oxidation of the cofactor,

present. (h) shows the negligible spectrum obtained with the use of inactivated molybdenum cofactor in the presence of thiophenol, as in (g). (i) shows a spectrum obtained by replacing the molybdenum cofactor with Na_2MoO_4 in aqueous dimethyl sulphoxide, in the presence of thiophenol, as in (g). (Note that the concentration of molybdenum was $250 \mu\text{M}$ in this experiment, compared with approx. $16 \mu\text{M}$ for all other samples.) (j) shows the spectrum of $[\text{Mo}(\text{SC}_6\text{H}_5)_4]^-$ in acetonitrile/dimethylformamide (4:1, v/v). Spectra are plotted on a *g*-value scale. Recording conditions were microwave power 20–30 mW, modulation amplitude 0.4 mT (a–f) or 0.16 mT (g–j), temperature approx. 120 K, and microwave frequency 9.3 GHz. The amplification used was similar for all samples ($\times 1$) except where indicated. Note that in (b), (e) and (f), in addition to the signals from molybdenum, small amounts of contaminating signals are also present in the free-radical region.

in dimethyl sulphoxide solution, by the procedures described in the Materials and methods section. Signals 1 and 2 are illustrated in Fig. 1 (see particularly *a*, *d* and *e*) and their *g*-values are listed in Table 3 (see below). Signal 1 was obtained by brief, and signal 2 by more prolonged, incubation of the molybdenum cofactor at apparent potentials in the region -50 mV to -150 mV . Signal 1 is axial in form, and signal 2 is rhombic; neither signal shows obvious indications of hyperfine coupling to ligand atoms. Minor variability in the parameters of signal 1 is considered below. If inactivated molybdenum cofactor, prepared (see the Materials and methods section) by deliberate exposure to air, was employed in place of the active material, then only very weak and ill-defined e.p.r. signals were observed (Fig. 1*f*).

We found that addition of thiophenol (final concentration 20–100 mM) to samples of the molybdenum cofactor giving signal 1 caused changes in the form of the signal. Appearance of the new signal (signal 1_{tp}) was accompanied by an increase in signal intensity (up to 65–85% of the molybdenum; see Table 3 below), particularly when it was generated (see the Materials and methods section) with thiophenol present at the outset. As shown in Fig. 1(*g*), the general form of signal 1_{tp} is

not unlike that of signal 1 (Figs. 1*a*–1*c*). However, the linewidth is less and the *g*-values are rather different (Table 3), with the value of $g_{\parallel}$ shifted by about 0.003 to higher field. Replacing thiophenol by equivalent concentrations of phenol had no comparable effect on signal 1; if inactivated cofactor was used in place of active form, then only negligible signals were observed (Fig. 1*h*). Addition of thiophenol to molybdenum cofactor solutions giving signal 2 did not result in any change in this signal. (With thiophenol present initially, signal 2 could not be generated.)

Several additional signals not related to molybdenum were also encountered, usually in very small amounts. Weak signals, presumably due to free radicals, were sometimes apparent at $g = 2.00$ – 2.01 (e.g. Figs. 1*b*, 1*e* and 1*f*). Another signal was sometimes obtained on brief incubation of the molybdenum cofactor (prepared in the normal way), at the low potential of about -270 mV . This signal had a well-defined rhombic form, with *g*-values of 2.0319, 2.0119 and 2.0027 ($g_{\text{av.}}$ 2.0155). Though intensity was sometimes relatively high (corresponding to up to 10% of the molybdenum), the *g*-values appear too high for an Mo(V) complex, and it was confirmed that the signal was not due to this metal, by examination of the spectrum

Table 2. Range of conditions under which the various e.p.r. signals from the molybdenum cofactor were observed

General procedures for generating the signals by treatment of the active molybdenum cofactor at controlled potentials are described in the Materials and methods section. A '+' sign in one of the columns corresponding to e.p.r. signal 1, 1_{tp} or 2 indicates that the signal was observed under the conditions indicated; a '-' sign indicates that no signal was observed, and a blank indicates that no experiment was carried out. Abbreviations and conditions were as follows: enzymes were xanthine oxidase (XO), desulpho xanthine oxidase [XO(ds)] and sulphite oxidase (SO); solvents were dimethyl sulphoxide containing 3–8% (v/v) water (DMSO), *N*-methylformamide containing 2–8% (v/v) water (NMF), or water (Aq); buffers were potassium phosphate buffer (5–20 mM) or Tris/HCl (5–20 mM) (both pH 7.8 in water); treatment with sodium dodecyl sulphate (SDS) or guanidinium chloride was carried out as described in the Materials and methods section; protein-free cofactor was obtained as described in Table 1, Expt. 2; mediator dyes were as listed in the Materials and methods section.

Enzyme	Solvent	Treatment	Buffer	Dyes	Oxidant	Signal 1	Signal 1 _{tp}	Signal 2
XO	DMSO	—	P _i	+	[Fe(CN) ₆] ³⁻	+	+	+
XO	DMSO	—	P _i	+	Air	+		
XO	DMSO	—	P _i	—	[Fe(CN) ₆] ³⁻	+		+
XO	DMSO		Tris	+	[Fe(CN) ₆] ³⁻	+	+	—
XO (ds)	DMSO		P _i	+	[Fe(CN) ₆] ³⁻	+	+	+
XO	Aq	Guanidine	P _i	+	[Fe(CN) ₆] ³⁻	—	—	—
XO	Aq	SDS	P _i	+	[Fe(CN) ₆] ³⁻	—	—	—
XO	DMSO	SDS	P _i	+	[Fe(CN) ₆] ³⁻	+	+	+
XO	DMSO	Protein-free	P _i	+	[Fe(CN) ₆] ³⁻	+	+	+
XO	NMF	—	P _i	+	[Fe(CN) ₆] ³⁻	+		—
XO	NMF	—	Tris	—	[Fe(CN) ₆] ³⁻	+		—
XO	NMF	—	Tris	+	[Fe(CN) ₆] ³⁻	+	+	—
SO	DMSO	—	P _i	+	[Fe(CN) ₆] ³⁻	+	+	—

* Parameters of the signal ($g_{\perp} = 1.9793$) were slightly different from those given in Table 3.

† Parameters of the signal ($g_{\parallel} = 2.0270$ – 2.0280) were slightly different from those given in Table 3; this is believed to be due to incomplete formation of the thiophenol complex (see the text).

‡ Parameters of the signal ($g_{\perp} = 2.0305$, $g_{\parallel} = 1.9770$) were slightly different from those given in Table 3.

of molybdenum cofactor prepared from xanthine oxidase enriched in ^{95}Mo . The origin of this signal is uncertain, though a sulphur-centred radical presumably from the protein is perhaps a possibility.

Range of conditions under which signals 1, 2 and 1_{ip} are obtainable

The three main signals could be obtained under a considerable variety of conditions, as summarized in Table 2, in addition to those considered so far and described in the Materials and methods section. Thus ability of molybdenum cofactor samples to yield signal 1 was not dependent on the presence of a particular buffer, organic solvent or oxidizing agent, or on the presence of the mediator dyes. Similar considerations applied also to signal 1_{ip} , though we investigated this less fully. Both of these signals were obtainable not only with unpurified molybdenum cofactor extracts from active xanthine oxidase but also with similar extracts from the desulpho form of this enzyme, or from sulphite oxidase. In the case of xanthine oxidase, the signals could also be obtained from protein-free molybdenum cofactor samples purified as described above (Table 1, Expt. 2). (Small variations in parameters of the signals under some conditions, noted in footnotes to Table 2, are considered later below.) In contrast with signals 1 and 1_{ip} , signal 2 seemed to be obtainable only under a more-limited range of conditions, in the presence of phosphate buffer and dimethyl sulphoxide.

It is particularly noteworthy (Table 2) that we failed to generate any of the three signals if the organic solvent, dimethyl sulphoxide or *N*-methylformamide, was omitted from the solution, as when molybdenum cofactor extracts were prepared (Hawkes & Bray, 1984) by denaturation of xanthine oxidase with sodium dodecyl sulphate or guanidinium chloride. The only signal we saw in such samples (oxidized at a range of potentials between -450 and -30 mV) was broad, featureless and symmetrical, and centred at $g = 1.941$, with a peak-to-peak width (on the derivative spectrum) of 4.5 mT. However, an almost identical signal could be generated under these conditions from Na_2MoO_4 in the absence of cofactor, thus clearly indicating that formation of this signal was due to the release of molybdenum from the cofactor. We confirmed that failure to obtain signals 1, 2 and 1_{ip} in aqueous solution was due to absence of the organic solvent from the medium. We were able to demonstrate this (Table 2), by first denaturing the enzyme in concentrated solution with sodium dodecyl sulphate, then diluting the molybdenum cofactor extract so obtained with dimethyl sulphoxide, before oxidizing it in the normal manner to generate the signals.

Incomplete formation of signal 1_{ip} : variability in the parameters of signal 1

We postulated that signal 1_{ip} might represent a complex of the molybdenum cofactor with thiophenol, with the thiol ligated to the metal. If this were the case, then incomplete replacement of signal 1 by signal 1_{ip} might be expected at relatively low concentrations of thiophenol. In support of this hypothesis, such an effect is clearly visible in Figs. 2(a)–2(c), in which the $g_{\parallel}$ features, only, of spectra from the cofactor are shown on an expanded scale. In the spectrum of Fig. 2(b), which was obtained with 10 mM-thiophenol, the g -value of the peak is intermediate between that of signal 1 (shown in the spectrum of Fig. 2(a), and that of signal 1_{ip} , fully developed with 100 mM-thiophenol and illustrated in the spectrum of Fig. 2(c). Close examination of the spectrum of Fig. 2(b) reveals, moreover, that the lineshape is consistent with a mixture of the spectra of Figs. 2(a) and 2(c). Also, in the $g_{\perp}$ region, corresponding behaviour was observed (results not shown). This spectral feature is

Fig. 2. $g_{\parallel}$ feature of e.p.r. spectra from the molybdenum cofactor

Spectra are plotted on a g -value scale, expanded to show the $g_{\parallel}$ feature, only. (a) shows the spectrum of signal 1, as in Fig. 1(a). (c) shows signal 1_{ip} obtained in the presence of 100 mM-thiophenol, as in Fig. 1(g). (b) shows a spectrum intermediate between (a) and (c) and was obtained by using a low concentration of thiophenol (10 mM). (d) is the spectrum obtained from Na_2MoO_4 in aqueous dimethyl sulphoxide in the presence of 100 mM-thiophenol, as in Fig. 1(i). (e) is the spectrum of $[\text{Mo}(\text{SC}_6\text{H}_5)_4]^-$ in acetonitrile/dimethylformamide, as in Fig. 1(j). E.p.r. conditions were as in Fig. 1 but with modulation amplitudes of 0.25 mT in (a)–(c) and (e) and 0.16 mT in (d).

sharp for signal 1_{ip} (peak-to-peak width on the derivative spectrum 0.90 mT) and broad for signal 1 (about 1.45 mT), with intermediate values (e.g. 1.05 mT) observed for samples (as in Fig. 2b) that showed mixed species. Formation of the complex of thiophenol with the molybdenum cofactor apparently did not take place instantaneously, since, in experiments with 5 mM-thiophenol, the proportion of signal 1_{ip} relative to signal 1 in the spectra was found to increase up to 10 min reaction time (at about -100 mV; preincubation at about -650 mV did not affect the time course of this process).

From the above data, apparently anomalous parameters for signal 1_{ip} from molybdenum cofactor prepared from xanthine oxidase with Tris buffer in *N*-methylformamide (Table 2, footnote) may be interpreted as being due to decreased molybdenum cofactor affinity for thiophenol in this medium. On the other hand, the comparable finding for molybdenum cofactor from sulphite oxidase, in this case under the usual conditions in dimethyl sulphoxide (Table 2, footnote), might be thought, tentatively, to indicate that a protein

ligand of the molybdenum has to dissociate, before association with thiophenol takes place, and that such dissociation occurs more readily for molybdenum cofactor from xanthine oxidase than for that from sulphite oxidase.

The gel-filtration experiments described above indicate that there is association, in our extracts, between molybdenum cofactor and residual protein molecules and that, in relation to such association, our preparations are likely to be non-homogeneous. In keeping with this, signal 1 shows indications of representing a family of very closely related signals, rather than a single signal. Evidence for this is summarized in Tables 2 (footnotes) and 3, with representative spectra illustrated in Figs. 1(a)–1(c). Even under the usual conditions of developing signal 1, its parameters were rather variable (standard deviation of g -values, on six samples, equal to expected upper limit of g -value measurement error). With other samples, larger g -value shifts were observed, e.g. for signal 1 from molybdenum cofactor from sulphite oxidase, the g -value of the $g_{||}$ peak was displaced to lower field by 0.0013, relative to that from xanthine oxidase.

Table 3. Parameters of the various e.p.r. signals from the molybdenum cofactor and correlation of signal intensities with cofactor activity

E.p.r. signals were generated from freshly prepared molybdenum cofactor and evaluated as described in the text. Signal intensities are expressed as percentage conversion of molybdenum in the samples into the signal-giving species (see the text for discussion of errors). The solvent was dimethyl sulphoxide unless otherwise indicated. Data on a model compound, and also on MoO_4^{2-} treated with thiols similarly to the cofactor, are shown for comparison (see the text for details). Assays of cofactor activity of the e.p.r. samples were carried out (see the Materials and methods section) both without (A_0) and with (A_m) the addition of MoO_4^{2-} to the complementation medium, and are expressed relative to activities of the untreated molybdenum cofactor samples. Where a range of e.p.r. signal intensities is indicated, the cofactor activity listed corresponds to samples showing the strongest signals. Corrected dependence on MoO_4^{2-} , $[100(1 - A_0/A_m) - 20] > 0$, indicates that some cofactor molecules have lost molybdenum, but remain capable of being re-activated in the complementation reaction by MoO_4^{2-} . [The correction factor of 20, deduced from the data of Hawkes & Bray (1984), makes an approximate allowance for dissociation of molybdenum from the cofactor during the assay.]

Sample/Signal	g_1 ($g_{ }$)	g_2	g_3 ($g_{\perp}$)	$g_{av.}$	Signal intensity (%)	Maximum activity remaining (A_0) (%)	Corrected dependence on MoO_4^{2-} [$100(1 - A_0/A_m) - 20$] (%)
Cofactor signal 1	2.0292 (± 0.0003)*		1.9776 (± 0.0003)*	1.9948	20–45	80 ± 8	0
Cofactor signal 1_{ip}	2.0258		1.9793	1.9948	65–85†	80 ± 8	0
$[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$	2.0182‡		1.9814‡	1.9938‡	—	—	—
Cofactor signal 2	1.9776	1.9590	1.9407	1.9591	10–25	60–75	0
Cofactor signal 1_{me}	2.0075	1.9769	1.9687	1.9840	10§	approx. 55	23
Cofactor signal 1_{ed}	2.0442 (± 0.0006)	1.9825	1.9790	2.0019	90	10	40
MoO_4^{2-} + ethanedithiol	2.0508	1.9817	1.9774	2.0033	95	—	—

* S.D. ($n = 6$).

† See Fig. 5 for conditions of generating the most intense signal.

‡ These values differ slightly from, and may be more accurate than, those given by Hanson *et al.* (1981). Identical g -values were obtained whether the spectrum was generated by dissolving $\text{NH}_4\text{Et}_3[\text{MoO}(\text{SC}_6\text{H}_5)_4]$ in acetonitrile/dimethylformamide (4:1, v/v) or by controlled oxidation/reduction of Na_2MoO_4 in dimethyl sulphoxide in the presence of thiophenol, as in the generation of signal 1_{ip} .

§ Signal 1 was also present; see Fig. 5.

Parameters of signals 1 and 1_{tp} in relation to those from model compounds

Parameters of all our signals are summarized in Table 3. Signals 1 and 1_{tp} both show axial symmetry with, for Mo(V) compounds, relatively high values of $g_{av.}$, and with $g_{\parallel}$ considerably above the free-electron value. This is in agreement with expectations (Meriwether *et al.*, 1966) for complexes with sulphur ligands, and, indeed, parameters of either signal are not unlike those of the well-characterized (Bradbury *et al.*, 1978) tetrathiolato anion $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$, for which data are included in Table 3.

The possibility that addition of thiophenol to the molybdenum cofactor results, in our experiments, in removal of the metal from the cofactor and formation of $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$ may, however, readily be excluded, for a number of reasons (including some based on activity measurements discussed in the next section). Firstly, the g -values of signal 1_{tp}

are quite considerably different from those of the tetrathiolato complex (Table 3; also see spectra of the complex in Figs. 1*i*, and 1*j*, 2*d* and 2*e*). This applies also to the $A(^{95}\text{Mo})$ -values, as we found by preparing molybdenum cofactor substituted with this isotope. Spectra of signal 1_{tp} and $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$, compared in Figs. 3(*b*) and 3(*c*), are clearly distinct from one another, even when the narrower linewidth in the spectrum of the complex is taken into account. Also, as already noted, treatment of inactivated molybdenum cofactor (16 μM), from which the metal is known (Hawkes & Bray, 1984) to have dissociated, with thiophenol under the conditions used to generate signal 1_{tp} , failed to give any significant signal (Fig. 1*h*). In contrast, the signal of $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$ was readily generated (Fig. 1*i*), by treating Na_2MoO_4 , but at a distinctly higher concentration (250 μM), with thiophenol, in dimethyl sulphoxide under the standard conditions used to generate signals from the molybdenum cofactor.

Fig. 3. E.p.r. spectra of samples enriched with ^{95}Mo

(*a*) shows signal 1 from molybdenum cofactor from xanthine oxidase enriched with ^{95}Mo , generated under standard conditions, as in Fig. 1(*a*). (*b*) shows signal 1_{tp} derived from enriched xanthine oxidase. (*c*) shows $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$ in acetonitrile/dimethylformamide (4:1, v/v). Enrichment with ^{95}Mo corresponds to about 63% in all cases. Running conditions were as in Fig. 1 with modulation amplitude 0.4 mT (*a* and *b*) and 0.25 mT (*c*). The arrow corresponds to $g = 2.0037$.

From the above considerations and those in the previous sub-section, it seems that if signal 1 is to be regarded as a family of signals, then signal 1_{tp} is best thought of as a special member of that family. This point is brought out not only by the relative similarity of their g -values (Table 3; note particularly the identical g_{av} -values) but also by the considerable similarity of their ^{95}Mo spectra (see Figs. 3a and 3b).

Relationship of signals 1, 1_{tp} and 2 to the active cofactor

We used two approaches in deciding whether the various signals are related to the active molybdenum cofactor or otherwise. In the first, we measured the intensity of signals developed from freshly prepared molybdenum cofactor, in terms of the percentage conversion of molybdenum in the samples into the signal-giving species. (It needs to be stated that absolute, as opposed to relative, accuracy of the e.p.r. intensity measurements is probably only of the order of $\pm 20\%$ of the values quoted, mainly because of the rather large and somewhat uncertain corrections that have to be applied, as detailed in the Materials and methods section.) We also measured cofactor activities, with and without the addition of molybdate (see the Materials and methods section), on samples removed from e.p.r. tubes. Results of these measurements are summarized in Table 3. If, as the data indicate for signal 1_{tp} , most of the molybdenum is in the signal-giving species, and the activity remaining is also high, paralleling the signal intensity, and independent of added molybdate, then this is strong evidence that this signal arises from active molybdenum cofactor. For signal 2 on the other hand, maximum signal intensity (25%) is equal to or less than the percentage loss of activity (25–40%) shown by samples giving the signal. Thus it could well be that this signal represents a degradation product of the molybdenum cofactor.

For signal 1, activity remained high (80%) but intensity was relatively weak (20–45%), making it difficult to decide whether the signal is related to active cofactor or not [bearing in mind the possible equilibria among Mo(IV)-, Mo(V)- and Mo(VI)-containing species]. To obtain further information we therefore used an alternative approach, carrying out the degradation experiment illustrated in Fig. 4. In this, the time course of oxidative inactivation of the molybdenum cofactor was followed by removing samples at intervals for activity and for e.p.r. measurements. Activity, and intensity of signals 1 and 1_{tp} , all declined at roughly comparable rates, confirming that signal 1_{tp} is due to active molybdenum cofactor and establishing the same point in relation to signal 1.

It should be noted that our practice (see the

Fig. 4. Correlation of loss of cofactor activity with disappearance of e.p.r. signals 1 and 1_{tp} during oxidative inactivation of the molybdenum cofactor

Oxidative inactivation of freshly prepared molybdenum cofactor ($14\mu\text{M}$) was carried out by maintaining it at a potential of $-100 \pm 100\text{ mV}$ at $20\text{--}25^\circ\text{C}$ for the cumulative times indicated. At intervals the potential was briefly changed. Firstly, it was lowered to about -650 mV , which eliminated all e.p.r. signals. Samples for assay could then conveniently be removed. The potential was then raised again to generate signal 1 (●) (see the Materials and methods section for details of procedure), and the experiment was continued. Signal 1_{tp} (■) was generated subsequently in the samples in the e.p.r. tubes (see the Materials and methods section). Cofactor activity measurements (without addition of molybdate) were performed either on samples withdrawn, as above, from the reaction mixture independently of the e.p.r. measurements (□) or on samples from the e.p.r. tubes after both the spectra had been recorded (○). The 100% activity corresponds to the cofactor activity at the start of the experiment. Integrated e.p.r. signal intensities are expressed as a percentage of the molybdenum in the sample appearing in the signal-giving state for the signal in question. (Note that in many of the samples not containing thiophenol signal 2 was present in addition to signal 1.)

Materials and methods section) of diluting the molybdenum cofactor samples taken from e.p.r. tubes into $\text{Na}_2\text{S}_2\text{O}_4$ -containing dimethyl sulphoxide before measuring their cofactor activity raises the possibility that the resulting re-reduction might, conceivably, cause free Mo to become rebound to the cofactor. Thus low molybdate-dependence of the cofactor activity (see Table 3 caption) might, in this case, give a misleading impression of molybdenum in the Mo(V) signal-giving species having remained associated with the cofactor. However, for both signals 1 and 1_{tp} , experiments (results not shown) with low concentrations of molybdenum cofactor ($2\text{--}3\mu\text{M}$), in which sam-

ples were taken from the oxidized signal-giving solutions and added directly, without prior reduction, to cofactor assay mixtures, yielded results indistinguishable from those in Table 3, thereby excluding the above possibility.

Signals obtained in the presence of other thiols

In extension of our studies with thiophenol, we carried out experiments in the presence of two other thiols, mercaptoethanol and ethanediol. However, as is shown in Table 3, incubation in the presence of either, but especially the latter, caused more extensive loss of cofactor activity than was associated with development of signals 1 or 1_{tp}.

Fig. 5 shows e.p.r. spectra for the active and inactive molybdenum cofactor in the presence of each of these thiols. Spectra were generated in the usual way, in dimethyl sulphoxide with the thiols present. For the active molybdenum cofactor, mercaptoethanol gave partial conversion (Fig. 5b) into a new signal-giving species (Fig. 5c), designated signal 1_{me} (see Table 3 for parameters). Inactivated molybdenum cofactor, with this thiol, gave only a weak and ill-defined signal (Fig. 5d). Some cofactor activity was lost in developing signal 1_{me}. It is particularly interesting to note (Table 3) the increased molybdate-dependence of the residual activity, which implies that mercaptoethanol has, unlike thiophenol, a tendency to remove the metal from the cofactor.

The dithiol, ethanediol, gave strong e.p.r. signals both for the active molybdenum cofactor (signal 1_{ed}; see Fig. 5e, and Table 3 for parameters), and from the inactivated form (Fig. 5f), these signals differing from one another only slightly, though significantly (especially in the $g_{\parallel}$ region). Development of signal 1_{ed} was accompanied by loss of all but a small amount of the molybdenum cofactor activity (Table 3), and by increased molybdate-dependence of the residual activity. On subjecting Na₂MoO₄ to the signal-generating procedure in the presence of ethanedithiol, we obtained a signal (not illustrated) indistinguishable from that from the inactivated molybdenum cofactor (Fig. 5f). The parameters of this signal (Table 3) are in agreement with those for the complex of molybdenum(V) with this thiol (Meriwether *et al.*, 1966; Kikuchi *et al.*, 1982), which have been confirmed independently in this laboratory (G. N. George, unpublished work). Thus this dithiol, unlike the two monothiols that we used, binds molybdenum from the inactivated cofactor and converts it in high yield into the free Mo(V) complex.

Discussion

Though the purification work on the molybdenum cofactor has not been concluded, the

Fig. 5. Effect of ethanedithiol and mercaptoethanol on the e.p.r. spectrum of the molybdenum cofactor

(a) shows signal 1 generated in the normal way without addition of thiols, as in Fig. 1(a). (b) and (e) correspond to signals generated similarly but in the presence of 25 mM-mercaptoethanol or approx. 80 mM-ethanedithiol respectively. (e) corresponds to the pure signal 1_{ed}, but (b) is signal 1_{me} contaminated with signal 1. Thus (c) is a difference spectrum [obtained from (a) and (b)] corresponding to pure signal 1_{me}. (d) and (f) are spectra from inactivated molybdenum cofactor, generated respectively in the presence of mercaptoethanol and ethanedithiol. In (d) the amplification has been increased 10-fold. E.p.r. conditions were as in Fig. 1, with modulation amplitude 0.4 mT. The arrow corresponds to $g = 2.0037$.

studies reported appear to point the way for further investigations. They also provide information important in interpreting the e.p.r. results.

Fig. 6 shows diagrammatically the structures that we propose for the signal-giving species of signals 1 and 1_{tp}. We propose, from the axial symmetry of the spectra and from the g -values, that

Fig. 6. Schematic representation of the proposed environment of Mo in the cofactor in various e.p.r. signal-giving species

The metal is shown co-ordinated equatorially to four thiolate groups and axially to a terminal oxygen atom. Two of the thiolate groups are supposed to come from the cofactor (shown as an elliptical shape) and the others, initially, from cysteine residues in the protein. Thus (a) corresponds to cofactor bound to a relatively large peptide, whereas in (b) it is bound to two smaller peptides, and in (c) the peptides have been displaced by thiophenol. With the metal in the Mo(V) state, (a) and (b) would give variants of signal 1, and (c) would give signal 1_{tp}.

their structures are both basically the same as that of the $[\text{MoO}(\text{SC}_6\text{H}_5)_4]^-$ ion. This ion (Bradbury *et al.*, 1978) has square pyramidal symmetry about molybdenum, with the molybdenum atom 0.082 nm above the plane formed by the four sulphur ligand atoms, with the terminal oxygen ligand above the molybdenum. Final interpretation (*A*-values and angles of non-coincidence of the axes) of the ^{95}Mo e.p.r. spectra of signals 1 and 1_{tp} (Fig. 3a and 3b) in comparison with the model compound (Fig. 3c; see Hanson *et al.*, 1981), should eventually provide more information on the geometry of the signal-giving species. Until this has

been done, the structures given in Fig. 6 are obviously tentative. In our earlier work (Hawkes & Bray, 1984) the effect of thiols on molybdenum-dependence of the cofactor assay also provided evidence for ligation of molybdenum in the cofactor to thiolate groups. Because of the tightness of the binding of molybdenum to the cofactor in the reduced state, as reported by these workers, it is necessary to assume that the as yet ill-defined organic part of the cofactor molecules provides at least two of the four proposed thiolate ligands. Thus we suggest (Figs. 6a and 6b) that, in the cofactor in crude extracts, two of the thiolate ligands of the molybdenum are provided by the cofactor molecule itself, with the other two provided by cysteine residues from the protein. Precisely which cysteine residues these would be would depend on the detailed history of the sample. This would account for the slight variability of the parameters of signal 1.

In the signal-giving species of signal 1_{tp} (Fig. 6c) the protein ligands have been replaced by thiophenol molecules. This species is obtained, with almost quantitative conversion of molybdenum into the Mo(V) state, on treatment of the cofactor with an excess of thiophenol. The ligand exchange appears to take place within a few minutes, at most, at an apparent potential of -100 mV . (The indications are that exchange is rather slower at lower apparent redox potentials.) There is little loss of cofactor activity in the presence of thiophenol. Thiophenol residues must therefore dissociate from the molybdenum cofactor quite readily in the assay, to be replaced presumably by thiolate ligands from the apo nitrate reductase molecule. (Note that it should in principle be possible to determine the number of thiophenol ligands in the signal-giving species by the use of thiophenol enriched with ^{33}S .)

Behaviour of the molybdenum cofactor in the presence of two other thiols contrasted sharply with that in the presence of thiophenol. Mercaptoethanol formed in rather low yield a complex with distinct e.p.r. parameters. The lower *g*-values suggest that there are fewer sulphur ligands in this species than in the species of signals 1 and 1_{tp}. Assuming signal 1_{me} arises from the cofactor, it is possible that the oxygen and the sulphur of the mercaptoethanol molecule are replacing two cysteine ligands.

The dithiol ethanedithiol is notably efficient in complexing the molybdenum liberated from O₂-inactivated cofactor. However, it appears to form a discrete complex with originally active molybdenum cofactor molecules. Possibly the structure of this is analogous to that of the complex with thiophenol but having one ethanedithiol molecule taking the place of two thiophenol molecules. If this is accepted, it is then necessary, in order to ex-

plain the very low activity in the presence of ethanedithiol, to assume that apo nitrate reductase cannot compete effectively for the cofactor with the chelating ethanedithiol ligand. However, further work would be required to establish more fully the mechanism of action of ethanedithiol and of mercaptoethanol on the molybdenum cofactor.

The structure of the species giving rise to signal 2 is uncertain. This has quite low g -values and presumably has fewer sulphur ligands than do the other species. It may well represent a degradation product of active molybdenum cofactor. Perhaps one of the sulphur ligands of the cofactor, as well as the sulphur ligands derived from the protein, have dissociated. Possibly, ligation of a phosphate group is involved, since this seemed to be required for the generation of the signal-giving species.

The purification work that we have carried out, in conjunction with our studies on the thiophenol complex, suggest a possible method for further purification work on the active molybdenum cofactor. Clearly direct removal of protein and of peptides is not a simple matter and probably requires breakage of thiolate bonds to the molybdenum. Oxidation by air would involve concomitant irreversible degradation of most of the cofactor (Hawkes & Bray, 1984). However, our work suggests that conversion of the molybdenum cofactor into the thiophenol complex could, with little loss of activity, break the association with peptides and thus facilitate purification. Though it may well be that further investigations with a wider range of thiols would reveal that other thiols could be substituted for thiophenol, nevertheless it would seem that its affinity for the molybdenum cofactor is ideally suited for our purpose. Thus affinity of thiophenol is sufficiently high that it can displace protein ligands, yet sufficiently low that, on dilution in the presence of apo nitrate reductase, it can be displaced once again for the assay.

In conclusion, we note that e.p.r.-active species from all of the molybdoenzymes show strong coupling of protons to molybdenum. These protons are on $-OH$ or $-SH$ groups ligated to the metal. In contrast, e.p.r.-active species from the cofactor show no evidence for the presence of such strongly coupled protons. We can only speculate as to why this should be. The most likely interpretation is that the proteins in every case impose an unusual co-ordination geometry on to the metal (which is no doubt related to the catalytic activity of the enzymes), probably by forcing a protonatable oxygen (or sulphur) ligand into an equatorial position.

We thank Dr. G. N. George and Dr. R. Durrant for supplying compounds, and the former for discussions on the e.p.r. parameters. The work was supported by the Wellcome Trust and by the Medical Research Council.

References

- Aasa, R. & Vänngård, T. (1975) *J. Magn. Reson.* **19**, 308–315
- Alikulov, Z. A., L'vov, N. P., Burkhanov, S. S. & Ketrovich, V. L. (1980) *Izv. Akad. Nauk SSSR Ser. Biol.* **5**, 712–718
- Boyd, I. W., Dance, I. G., Murray, K. S. & Wedd, A. G. (1978) *Aust. J. Chem.* **31**, 279–284
- Bradbury, J. R., Mackay, M. F. & Wedd, A. G. (1978) *Aust. J. Chem.* **31**, 2423–2430
- Bray, R. C. (1975) *Enzymes 3rd Ed.*, **12**, 229–419
- Bray, R. C. (1980a) in *Biological Magnetic Resonance* (Berliner, L. J. & Reuben, J., eds.), pp. 45–84, Plenum Press, New York
- Bray, R. C. (1980b) *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, New York
- Bray, R. C. & Meriwether, L. S. (1966) *Nature (London)* **212**, 467–469
- Bray, R. C., Barber, M. J. & Lowe, D. J. (1978) *Biochem. J.* **171**, 653–658
- Bray, R. C., George, G. N., Hawkes, T. R. & Morpeth, F. F. (1983) in *Recent Developments in the Chemistry of Chromium, Molybdenum and Tungsten* (Dilworth, J. R. & Lippert, M. F., eds.), p. L7, Royal Society of Chemistry (Dalton Division), London
- Cammack, R., Barber, M. J. & Bray, R. C. (1976) *Biochem. J.* **157**, 469–478
- Claassen, V. P., Oltmann, L. F., Van't Riet, J., Brinkman, U. A. Th. & Stouthamer, A. H. (1982) *FEBS Lett.* **142**, 133–137
- Cramer, S. P. & Minak, V. (1983) in *Recent Developments in the Chemistry of Chromium, Molybdenum and Tungsten* (Dilworth, J. R. & Lippert, M. F., eds.), p. P17, Royal Society of Chemistry (Dalton Division), London
- Hanson, G. R., Brunette, A. G., McDonnell, A. C., Murray, K. S. & Wedd, A. G. (1981) *J. Am. Chem. Soc.* **103**, 1953–1959
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Hawkes, T. R. & Bray, R. C. (1984) *Biochem. J.* **219**, 481–493
- Johnson, J. L. (1980) in *Molybdenum and Molybdenum-Containing Enzymes* (Coughlan, M. P., ed.), pp. 345–383, Pergamon Press, Oxford
- Johnson, J. L. & Rajagopalan, K. V. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6856–6860
- Johnson, J. L., Hainline, B. E. & Rajagopalan, K. V. (1980) *J. Biol. Chem.* **255**, 1783–1786
- Kikuchi, T., Sugiura, Y. & Hisashi, T. (1982) *Inorg. Chim. Acta* **66**, L5–L8
- Lamy, M. T., Gutteridge, S. & Bray, R. C. (1980) *Biochem. J.* **185**, 397–403
- Lee, K.-Y., Pan, S.-S., Erickson, R. & Nason, A. (1974) *J. Biol. Chem.* **249**, 3941–3952
- L'vov, N. P., Ganelin, V. L., Alikulov, Z. A. & Ketrovitch, V. L. (1975) *Izv. Akad. Nauk SSSR Ser. Biol.* **3**, 371–376
- Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598

Meriwether, L. S., Marzluff, W. F. & Hodgson, W. G. (1966) *Nature (London)* **212**, 465–467

Pateman, J. A., Cove, D. J., Rever, B. M. & Roberts, D. B. (1964) *Nature (London)* **201**, 58–60

Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **19**, 544–552

Swartz, H. M., Bolton, J. M. & Borg, D. C. (1972) *Biological Applications of Electron Spin Resonance*, pp. 98–101, Wiley-Interscience, New York