

Electron-Paramagnetic-Resonance Parameters of Molybdenum(V) in Sulphite Oxidase from Chicken Liver

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A study has been made of e.p.r. signals due to Mo(V) in reduced sulphite oxidase (EC 1.8.3.1) from chicken liver. Reduction by SO_3^{2-} , or photochemically in the presence of a deazaflavin derivative, produces spectra indistinguishable from one another. Three types of spectra from the enzyme were distinguished and shown to correspond to single chemical species, since they could be simulated at both 9 and 35 GHz by using the same parameters. These were the low-pH form of the enzyme, with g_{av} 1.9805, the high-pH form, with g_{av} 1.9681 and a phosphate complex, with g_{av} 1.9741. The low-pH form shows interaction with a single exchangeable proton, with $A(1\text{H})_{\text{av}}$ (hyperfine coupling constant) = 0.98 mT, probably in the form of an MoOH group. Parameters of the signals are compared with those for signals from xanthine oxidase and nitrate reductase. The signal from the phosphate complex of sulphite oxidase is unique among anion complexes of Mo-containing enzymes in showing no hyperfine coupling to protons. There is no evidence for additional weakly coupled protons or nitrogen nuclei in the sulphite oxidase signals. The possibility is considered that the enzymic mechanism involves abstraction of a proton and two electrons from HSO_3^- by a Mo=O group in the enzyme.

Liver sulphite oxidase (sulphite–oxygen oxidoreductase, EC 1.8.3.1) has been quite extensively studied since the enzyme was first isolated by Cohen & Fridovich (1971a). It contains one molecule of haem and one atom of Mo per subunit (Cohen & Fridovich, 1971b; Cohen *et al.*, 1971). Controlled proteolysis yields separate haem- and Mo-containing fragments (Johnson & Rajagopalan, 1977; Guiard & Lederer, 1977). The latter fragment retains sulphite-oxidizing ability, if $\text{Fe}(\text{CN})_6^{3-}$ or molecular O_2 is used as the electron acceptor (Johnson & Rajagopalan, 1977). On the other hand, the amino acid sequence of the haem fragment is analogous to that of the corresponding fragment of flavocytochrome b_2 from bakers' yeast, or of intact microsomal cytochrome b_3 (Guiard & Lederer, 1977).

Mo was discovered as a constituent of sulphite oxidase by e.p.r. spectroscopy (Cohen *et al.*, 1971),

Abbreviation used: e.x.a.f.s. ('EXAFS'), extended X-ray absorption fine structure.

and a number of subsequent studies on the enzyme used this technique (see, e.g., Kessler & Rajagopalan, 1972, 1974; Kessler *et al.*, 1974; Johnson & Rajagopalan, 1976). Nevertheless, at least in comparison with one other Mo-containing enzyme, namely xanthine oxidase (Bray, 1975, 1979; Gutteridge & Bray, 1979), e.p.r. has so far yielded relatively little detailed or quantitative information on the Mo of sulphite oxidase. On the other hand, since then, Cramer *et al.* (1979) have reported studies from e.x.a.f.s. measurements, providing preliminary information on the ligand environment of Mo in this enzyme.

In the present work we report parameters, obtained from measurements at 9 and at 35 GHz and with the help of computer simulations, for three Mo(V) e.p.r. signals from chicken liver sulphite oxidase. Such values should be considerably more reliable than previous ones (see, e.g., Cohen *et al.*, 1971), which were deduced simply by inspection of spectra at 9 GHz. We discuss the significance of some of the e.p.r. parameters.

Materials and Methods

Activity measurements and haem and Mo contents

Sulphite oxidase activity was measured spectrophotometrically with cytochrome *c* as electron acceptor (Cohen & Fridovich, 1971*a*). Final concentrations of 0.4 mM- Na_2SO_3 and 10 μM -ferricytochrome *c* in 25 mM-sodium 4-morpholinepropanesulphonate (Mops) buffer, pH 7.8, with 0.1 mM-EDTA, were used at 23.5°C. One unit of sulphite oxidase activity is defined as the quantity giving $\Delta A_{550\text{nm}}^{1\text{cm}}/\text{min} = 0.1$, under these conditions. Since SO_4^{2-} is known to inhibit sulphite oxidase (Cohen & Fridovich, 1971*a*), it was necessary to remove most of any $(\text{NH}_4)_2\text{SO}_4$ present in enzyme samples before carrying out assays. In our assay system in Mops buffer, 50% inhibition was obtained at an SO_4^{2-} concentration of about 20 mM. Specific activities are units of sulphite oxidase per mg of protein. Protein was determined by the method of Lowry *et al.* (1951).

Haem contents were determined from A_{413} , taking $\epsilon_{\text{mm}} = 99.9$ (Cohen & Fridovich, 1971*b*). Sulphite oxidase concentrations in partially purified preparations were calculated from the haem content, by assuming 1 haem group per 55 000 mol.wt. (Kessler & Rajagopalan, 1972). Mo was determined colorimetrically as described by Hart *et al.* (1970) on two samples of enzyme.

Preparation and properties of sulphite oxidase

A convenient procedure yielding partially purified sulphite oxidase was based on published methods (Cohen & Fridovich, 1971*a*; Kessler & Rajagopalan, 1972; Kessler *et al.*, 1974), but avoided both the preparation of an acetone-dried powder and subsequent low-temperature fractionation with acetone.

Frozen chicken livers were obtained from a local supermarket, and homogenized in 10 mM-potassium phosphate buffer, pH 7.8, containing 0.1 mM-EDTA. (EDTA at this concentration was added routinely to all solutions containing the enzyme throughout our work.) To the homogenate were added sodium deoxycholate (0.1%) and $(\text{NH}_4)_2\text{SO}_4$ (to 20% saturation), and the mixture was then heated briefly to 56°C. After centrifuging off the precipitate, the supernatant was twice fractionated with $(\text{NH}_4)_2\text{SO}_4$, between the limits of 20 and 50% saturation. After dialysis against 50 mM-potassium phosphate buffer, pH 7.8, the product was chromatographed on DEAE-cellulose (Whatman DE52, from Whatman Ltd., Maidstone, Kent, U.K.) by using a column (4.5 cm diam. \times 12.5 cm high) and eluting with a 50–200 mM gradient of the same buffer. Sulphite oxidase-containing fractions were combined.

The product was obtained in high yield (approx. 60 mg of sulphite oxidase per kg of liver) and was entirely suitable for e.p.r. work. It could be stored in-

definitely as frozen beads at the temperature of liquid N_2 . It was free from significant contamination with xanthine dehydrogenase, as judged by additions of purine failing to elicit an e.p.r. signal. However, our specific activity (300 to 500 units/mg) was much less than the best value (6694 units/mg) reported under slightly different assay conditions by Kessler & Rajagopalan (1972). Part of the discrepancy seems to be due to the presence of a demolybdo sulphite oxidase (cf. Johnson *et al.*, 1977) in our preparations. Thus we found Mo/haem ratios of about 0.4. Kessler & Rajagopalan (1972) did not report a Mo content for chicken liver sulphite oxidase, but Cohen *et al.* (1971) found a Mo/haem ratio of 1.05 for the bovine enzyme. Our samples appeared not to be contaminated by extraneous haem, since identical absorption spectra for the reduced enzyme (Cohen & Fridovich, 1971*b*) were obtained on reduction with either SO_3^{2-} or $\text{S}_2\text{O}_4^{2-}$. Our value (about 5) for the absorbance ratio A_{280}/A_{413} , in comparison with the best value of Kessler & Rajagopalan (1972) (0.60), suggests higher purity for our samples than do the specific-activity values. This is in keeping with contamination by the demolybdo-enzyme.

For some experiments we used an alternative preparation in which treatment of the homogenate with butan-1-ol replaced the heating step. $(\text{NH}_4)_2\text{SO}_4$ fractionation and DEAE-cellulose chromatography were as described above, and gel filtration (Sephadex G-100, from Pharmacia, London W5 5SS, U.K.) was introduced as an additional final step. The product had specific activity about 500 units/mg, but was obtained in lower yield.

E.p.r. measurements

Samples of sulphite oxidase, after dialysis into a suitable medium, were concentrated, usually to about 60 μM -haem, by ultrafiltration (PM10 membrane; Amicon, High Wycombe, Bucks., U.K.). Reduction of the enzyme with SO_3^{2-} (1 mM) aerobically for 1 min was generally used to elicit the e.p.r. signals. However, these conditions did not appear to be critical. For experiments in which reduction by SO_3^{2-} was replaced by photochemical reduction in the presence of a deazaflavin derivative (Massey & Hemmerich, 1977), the procedure was as follows. 10-Methyl-5-deazaflavin-3-propanesulphonate (potassium salt, 5 μM ; generously provided by Professor P. Hemmerich, University of Konstanz, Konstanz, Germany) was added to the enzyme, containing 1 mM-EDTA, in an e.p.r. tube, and irradiation was carried out anaerobically for 1 min by using a slide projector as a light source.

Many of the e.p.r. measurements reported below were performed on enzyme prepared by the butanol procedure. However, a number of these were repeated with the alternative purification method, and similar results were obtained. Transfer of the

enzyme into $^2\text{H}_2\text{O}$ was by dialysis and was monitored by n.m.r. Final residual $^1\text{H}_2\text{O}$ contents did not exceed 4%. pD in $^2\text{H}_2\text{O}$ refers to corrected readings obtained with a glass electrode.

E.p.r. spectra, at about 120K and at 9 or 35 GHz, were recorded on a Varian E9 spectrometer linked to a computer (Bray *et al.*, 1978). Modulation amplitude and microwave power at the two frequencies were respectively 0.2 and 1.0 mT and 5 mW and 10 decibels attenuation. Computer-generated difference spectra, where required, were obtained as described by Bray *et al.* (1978). Integration to obtain signal intensities was carried out on the computer, as described previously, with correction for the outer I (nuclear spin) = $5/2$ lines of the spectrum (Barber *et al.*, 1976). A cupric salt in the presence of EDTA was used as integration standard and the transition probability correction was made as described by Aasa & Vännegård (1975). Simulation of e.p.r. spectra was as described by Lowe (1978). ^1H splittings were taken to be 6.51 times smaller than ^1H splittings. For simulations at 35 GHz, 0.2 mT was added to half-linewidths used at 9 GHz.

Results

Cohen *et al.* (1971) presented e.p.r. spectra for a high-pH and for a low-pH form of bovine sulphite oxidase. Subsequently Kessler & Rajagopalan (1972) presented similar spectra for the chicken enzyme. Only the low-pH form shows coupling of exchangeable protons to Mo. Though g -values and $A(^1\text{H})$ values were reported (Cohen *et al.*, 1971), the low-pH signal was analysed in terms of axial symmetry, whereas its spectrum, particularly that in $^2\text{H}_2\text{O}$, is obviously rhombic in form.

Our results on the chicken liver enzyme are presented in Figs. 1, 2 and 3 and Table 1. Fig. 1 shows the low-pH spectrum in $^1\text{H}_2\text{O}$ and in $^2\text{H}_2\text{O}$, recorded at 9.3 and at 34.4 GHz. In all cases experimental and simulated spectra agree closely with one another. Apart from a conventional increase in the linewidth used in simulating the 35 GHz spectra (see the Materials and Methods section), a single set of parameters as listed in Table 1 was used in all four simulations. This is strong evidence that only a single species of Mo(V) in the enzyme is involved.

The high-pH spectrum in $^1\text{H}_2\text{O}$ is presented at the two frequencies in Figs. 2(a) and 2(e), along with the corresponding simulations (Figs. 2(b) and 2(f)), which are again in good agreement with the experimental spectra. In a further experiment (results not shown) we examined the high-pH spectrum in $^2\text{H}_2\text{O}$. In agreement with Cohen *et al.* (1971), we found no gross change in this solvent. However (and again in agreement with the data of these workers, though in their case they did not comment on it), $^2\text{H}_2\text{O}$ did cause an apparent slight sharpening of some features of the spectrum. We are not yet certain of the reason for this small change, though difference spectra suggested that it might be due to small amounts of another proton-split species. Simulations indicated, on the other hand, that there was probably no very weakly coupled proton (such as the second proton in the Slow signal from xanthine oxidase; Gutteridge *et al.*, 1978b), present in the high-pH form, giving broadening but no resolved splitting.

Sulphite oxidase is known to be inhibited by a number of anions at low or moderate concentrations (Cohen & Fridovich, 1971a; see also the Materials and Methods section). In keeping with this, Kessler & Rajagopalan (1972, 1974) reported

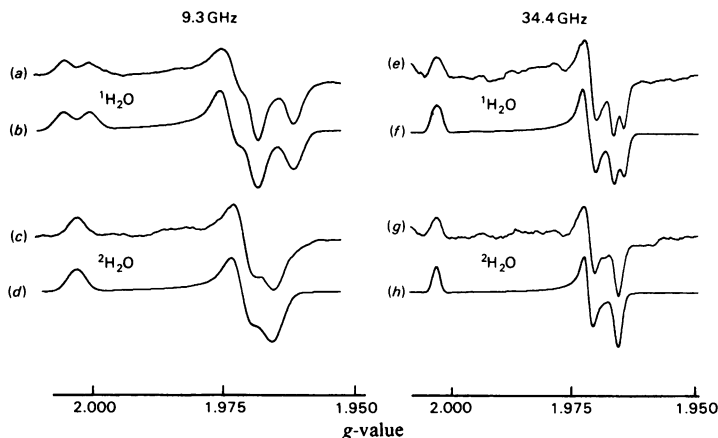


Fig. 1. The low-pH e.p.r. spectrum of sulphite oxidase $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ at 9.3 and 34.4 GHz

Spectra were obtained as described in the Materials and Methods section. (a), (e), (c) and (g) are experimental spectra obtained for samples in 100 mM-Mops buffer, pH or pD about 6.5. (b), (f), (d) and (h) are computer simulations, obtained by using the parameters given in Table 1.

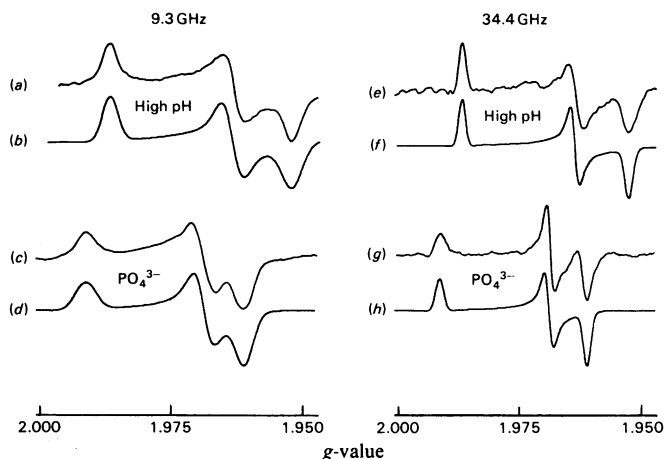


Fig. 2. The high-pH and the PO_4^{3-} e.p.r. spectra of sulphite oxidase at 9.3 and 34.4 GHz

Spectra were obtained in $^1\text{H}_2\text{O}$ as described in the Materials and Methods section. (a), (e), (c) and (g) are experimental spectra. (b), (f), (d) and (h) are computer simulations, obtained by using the parameters given in Table 1. The high-pH spectrum (a and e) was obtained in 100 mM-Caps [3-(cyclohexylamino)propanesulphonic acid sodium salt] buffer, pH about 10.0. The PO_4^{3-} spectrum (c and g) was obtained with 25 or 50 mM-potassium phosphate buffer, pH about 7.3. In the case of (g), a small amount of the high-pH spectrum (Fig. 1e) has been subtracted from the spectrum.

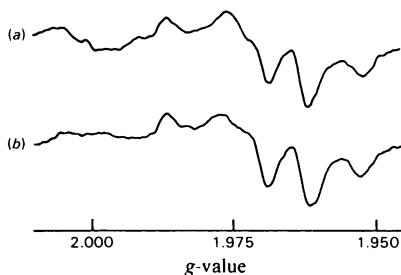


Fig. 3. Comparison at 9.3 GHz of the e.p.r. spectrum of sulphite oxidase reduced with SO_3^{2-} with that obtained on photochemical reduction in the presence of a deazaflavin derivative

(a) Shows the photochemically reduced sample and (b) the one reduced with SO_3^{2-} . Conditions of reduction were as described in the Materials and Methods section. Samples were in 100 mM-Mops buffer, pH about 7.0. Spectra are a mixture of the low- and the high-pH forms.

that a number of anions modify the e.p.r. spectrum of Mo(V) in sulphite oxidase. However, close inspection of the spectra presented by Kessler & Rajagopalan (1974) suggested to us that many of these, e.g. the ' SO_4^{2-} spectrum', were no more than mixtures of the high- and the low-pH spectral forms, whereas others differed little from the pure high-pH (CNS^- , F^-) or low-pH (Cl^- , NO_3^-) forms themselves. We therefore carried out a number of experiments, which will not be described in detail, on the effects of these anions on the spectrum of the enzyme at various pH values. In no case could we

distinguish any new species differing substantially in e.p.r. parameters from the low- and high-pH forms shown in Table 1.

However, in a number of enzyme samples containing PO_4^{3-} , and now in agreement with Kessler & Rajagopalan (1974), we distinguished a further e.p.r.-detectable species, quite distinct from either the high- or the low-pH form. We succeeded in obtaining spectra (Figs. 2c and 2g) in which the ' PO_4^{3-} signal' (as we shall term it) was the only species present, and we carried out simulations (Figs. 2d and 2h). Parameters are listed in Table 1. It is particularly noteworthy that the PO_4^{3-} signal is quite unchanged in $^2\text{H}_2\text{O}$ and thus shows no sign of proton splittings. Kessler & Rajagopalan (1974) presented a spectrum of the PO_4^{3-} signal similar to that of Fig. 2(c), but did not analyse it in any way.

PO_4^{3-} is a moderately strong inhibitor of sulphite oxidase activity (Cohen & Fridovich, 1971a). It is therefore not surprising that we frequently observed the PO_4^{3-} signal, sometimes along with the other signals, in enzyme samples prepared originally in PO_4^{3-} buffer, which had not been adequately dialysed to remove this ion.

Because of the anion effects on the enzyme, we thought it important to establish that SO_3^{2-} (or SO_4^{2-}) ions were not in any way modifying the spectra which we have described. In all work hitherto reported on sulphite oxidase, signals have been obtained by reduction with SO_3^{2-} . We therefore used, as an alternative, photochemical reduction in the presence of EDTA and catalytic amounts of a deazaflavin derivative, a general reduction procedure advocated

Table 1. E.p.r. parameters of Mo(V) in signals from sulphite oxidase and other molybdoenzymes

The values given for sulphite oxidase are those used in the simulations in Figs. 1 and 2; data for xanthine oxidase and for nitrate reductase are taken from the literature, for comparison. Proton hyperfine splittings and half-linewidths (Δ), at 9 GHz, are given in mT.

Enzyme	Signal	g-values			Proton splittings [A' (H)]			Δ_{av}	Reference		
		1	2	3	1	2	3			Av.	
Sulphite oxidase	Low-pH	2.0037	1.9720	1.9658	1.9805	0.85	0.80	1.30	0.98	0.27*	
	High-pH	1.9872	1.9641	1.9531	1.9681	—	—	—	—	0.29†	
	PO_4^{3-}	1.9917	1.9692	1.9614	1.9741	—	—	—	—	0.30‡	
Nitrate reductase	Low-pH	1.9989	1.9855	1.9628	1.9824	1.11	0.84	0.81	0.92	0.27	
	High-pH	1.9870	1.9805	1.9612	1.9762	—	—	—	—	0.30	
Xanthine oxidase	Rapid type 1§	1.9893	1.9703	1.9657	1.9751	{ 1.30 0.40	{ 1.39 0.30	{ 1.40 0.20	{ 1.36 0.30	0.16	Gutteridge <i>et al.</i> (1978 <i>a</i>)
	Rapid type 2	1.9951	1.9712	1.9616	1.9760	{ 1.07 1.07	{ 0.98 0.98	{ 0.93 0.93	{ 1.01 1.01	0.22	Bray <i>et al.</i> (1978)
	Slow	1.9719	1.9671	1.9551	1.9647	{ 1.66 0.16	{ 1.66 0.16	{ 1.56 0.16	{ 1.63 0.16	0.19	Gutteridge <i>et al.</i> (1978 <i>b</i>)

* Δ_1 , 0.28; Δ_2 , 0.25; Δ_3 , 0.27.† Δ_1 , 0.25; Δ_2 , 0.28; Δ_3 , 0.34.‡ Δ_1 , 0.33; Δ_2 , 0.27; Δ_3 , 0.29.

§ Parameters of the 1-methylxanthine complex are given.

|| Parameters of the xanthine complex are given.

by Massey & Hemmerich (1977). The spectrum so obtained (Fig. 3a) did not differ significantly from that obtained, under comparable conditions, on reduction of the enzyme by SO_3^{2-} (Fig. 3b); both are mixtures of the high- and low-pH spectra. Clearly, then, the presence of SO_3^{2-} or SO_4^{2-} is not essential for generation of either of these signals.

Integration of signals from sulphite oxidase obtained in several different experiments under various conditions indicated conversion of about 40–45% of the total to the signal-giving quinivalent state.

Discussion

Parameters of the three Mo(V) signals from sulphite oxidase are compared in Table 1 with those of some signals from xanthine oxidase (Gutteridge *et al.*, 1978*a,b*; Bray *et al.*, 1978) and from nitrate reductase (Vincent & Bray, 1978). A number of conclusions may be made.

The g_{av} values listed fall within a relatively narrow range (1.9647 to 1.9824), yet apart from their obviously differing catalytic properties, the three enzymes also differ in other important features, e.g. in the redox potentials of their Mo atoms (Vincent, 1979). If the metal in the three enzymes has different ligand atoms, then g -values do not provide any clear indication of this. However, it is noteworthy that, for nitrate reductase as well as for sulphite oxidase, g_{av} for the protonated low-pH form is higher than it is for the non-protonated high-pH form. Indeed, for sulphite oxidase the g_{av} value for the protonated form is one of the highest in Table 1, whereas g_{av} for the high-pH form is one of the lowest. Clearly, there must be a substantial structural change accompanying loss of the proton.

Linewidths in Table 1 are noteworthy, since high values might be indicative of unresolved hyperfine interactions with ligand atoms, particularly nitrogen. Nitrate reductase and sulphite oxidase show slightly higher linewidths than does xanthine oxidase. However, even for these two enzymes it would be difficult to accommodate in the linewidths nitrogen splittings much greater than 0.1 mT. We conclude that, if there are nitrogen ligands, their nuclei are only very weakly coupled to Mo.

We now turn to the proton splittings. We find only a single exchangeable proton coupled to the metal in sulphite oxidase. The enzyme, in this respect, is like nitrate reductase and contrasts with xanthine oxidase, where there are two coupled protons (Table 1). The value of $A(^1\text{H})_{\text{av}} = 0.98$ mT for the low-pH signal from sulphite oxidase is very similar to the corresponding value for nitrate reductase, but is smaller, for example, than that for the strongly

coupled proton of the Slow signal from desulpho xanthine oxidase, for which $A(^1\text{H})_{\text{av}} = 1.63$ mT. In the sulphite oxidase signal, the proton splitting is slightly more anisotropic than in the signals from the other enzymes; however, it is probably not sufficiently anisotropic to raise any serious question of the signs of the splittings being different.

It seems likely that all the strongly coupled protons in these enzymes (and perhaps also the more weakly coupled protons in two of the xanthine oxidase signals) are present in the form of MoOH or MoSH groups (cf. Gutteridge *et al.*, 1978*b*; Bray & Vänngård 1969). Terminal oxygen or terminal sulphur ligands of Mo in the oxidized enzymes could protonate on reduction of the metal to give these species. In sulphite oxidase, e.x.a.f.s. has provided evidence for the presence of terminal oxygen atoms in the oxidized enzyme (Cramer *et al.*, 1979). It is therefore most probable that the coupled proton is in the form of MoOH.

There is clearly a strong possibility, though direct evidence is lacking, that sulphite oxidase functions catalytically in a manner analogous to xanthine oxidase (Gutteridge *et al.*, 1978*a,b*), with the HSO_3^- ion transferring a proton to the terminal ligand of Mo and with two electrons going on to the metal atom.

In connection with the e.x.a.f.s. work on sulphite oxidase (Cramer *et al.*, 1979), our finding that, in the SO_3^{2-} -reduced enzyme, rather less than half of the Mo is in the quinivalent state, must cast doubt on conclusions drawn by these workers about the structure of the partially reduced enzyme. It seems that, under the conditions of the experiments reported, a mixture of Mo(IV), Mo(V) and Mo(VI) must have been present, making interpretation with regard to the structure in any one valence state of the metal premature. A similar conclusion has now been reached independently by S. P. Cramer (personal communication).

Finally, we turn to the PO_4^{3-} complex of sulphite oxidase (note that the state of ionization of the phosphate ion in the signal-giving species is quite unknown). This signal is unique among anion complexes described for molybdenum-containing enzymes (Vincent & Bray, 1978; Gutteridge *et al.*, 1978*a,b*; J. P. G. Malthouse, S. Gutteridge and R. C. Bray, unpublished work) in showing no proton hyperfine splitting. The significance of this observation, however, in terms of structure, conformation and ionization of the active centre of the enzyme remains to be determined. Further work will be required to determine precise values for the pK governing interconversion between the high- and low-pH forms of the enzyme (cf. Cohen *et al.*, 1971), to measure the dissociation constant for the phosphate complex and to assess possible effects of phosphate and other ions on the pK value. Why it

should be that PO_4^{3-} gives rise to an e.p.r.-detectable complex, whereas SO_4^{2-} does not do so, despite its showing inhibitory effects similar to those of PO_4^{3-} , is far from clear. Possibly SO_4^{2-} binds only to Mo(VI) or Mo(IV) in the enzyme, whereas PO_4^{3-} binds to Mo(V).

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