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The nature of the phosphate inhibitor complex of sulphite oxidase from electron-paramagnetic-resonance studies using oxygen-17

Steven GUTTERIDGE, M. Teresa LAMY and Robert C. BRAY
School of Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

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Studies of the effect of substitution with ^{17}O on the e.p.r. spectra at 9 and 35 GHz of Mo(V) in the phosphate complex of sulphite oxidase are reported. Substitution of ^{17}O-enriched water for normal water, for samples of the enzymes reduced by sulphite in the presence of normal phosphate, produced no detectable effect on the e.p.r. signal. If phosphate substituted with ^{17}O was used, coupling due to ^{17}O, producing large anisotropic splittings in the spectrum, was clearly detectable. It is concluded that phosphate is co-ordinated directly to molybdenum in the active site of the enzyme, in an equatorial type of ligand position. An oxygen ligand must be displaced from the molybdenum in the process of binding the phosphate. Implications concerning the mechanism of the enzymic reactions are discussed.

Studies of e.p.r. signals of molybdenum-containing enzymes are important in understanding the role of the metal in their reaction mechanisms and the structures of the molybdenum centres (see, e.g., Gutteridge & Bray, 1980*a*,*b*; Bray *et al.*, 1979; Bray, 1980). Although in the case of xanthine oxidase the precise structures of the various molybdenum(V) signal-giving species have not been fully determined, all of the signals have, nevertheless, been assigned to single enzymic species whose relationships to the catalytic processes seem reasonably clear (Bray, 1980). Until recently the molybdenum signals of sulphite oxidase were less well characterized. However, spectra of three separate species, generated by reduction of this enzyme under specific conditions with sulphite, have now been distinguished and studied at 9 and 35 GHz (Lamy *et al.*, 1980). One species with $g_{av.}$ 1.9805 is produced at moderately low pH and exhibits strong coupling of one exchangeable proton to molybdenum. A high-pH species has $g_{av.}$ 1.9681 and is without interacting protons. The third species ($g_{av.}$ 1.9741) is distinct from these and lacks proton splittings. It is detected only in the presence of phosphate, an inhibitor of sulphite oxidase activity.

The nature of the groups that bind the molybdenum in sulphite oxidase has been studied more directly with X-ray absorption spectroscopy (e.x.a.f.s.) (Cramer *et al.*, 1979*a*). In the oxidized enzyme it seems that the ligands are sulphur atoms together with two terminal oxygen atoms. On reduction to the Mo(IV) state, the ligands are essentially the same, except that one of the terminal oxygen atoms at 0.17 nm (1.7 Å) is replaced by a group at a longer distance of 0.206 nm (2.06 Å). Cramer *et al.* (1979*a*) suggested that this group is either oxygen or nitrogen, although protonation of the terminal oxygen group to, or its replacement by, a hydroxy group is not considered by these workers, yet is consistent with their data. It is consistent, also, with e.p.r. evidence (Lamy *et al.*, 1980) that, in the low-pH Mo(V) species, the exchangeable proton is present as Mo–OH.

Identification of oxygen atoms co-ordinated directly to the molybdenum, whether in the form of Mo=O or MO–OH, is important, because they could well be involved in the reaction mechanism of sulphite oxidase. E.p.r. studies using ^{17}O, a magnetic isotope having I (nuclear spin) $= \frac{5}{2}$, in the form of enriched water (Cramer *et al.*, 1979*b*; M. T. Lamy, S. Gutteridge & R. C. Bray, unpublished work) have provided confirmation that oxygen is bound close to the molybdenum. At least one exchangeable oxygen is strongly coupled to the metal in both the low- and high-pH signals. Because of the unique nature of the phosphate inhibitor complex of the enzyme, we thought it important to extend ^{17}O studies to this species.

Abbreviations used: e.x.a.f.s., extended X-ray absorption fine structure; Mes, 4-morpholine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

Materials and methods

Preparation of sulphite oxidase samples

Sulphite oxidase, partially purified from chicken

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liver (Lamy *et al.*, 1980), was dialysed against a 5mM-Mes/NaOH buffer, pH6.8, then freeze-dried and stored over liquid N_2. For use, freeze-dried enzyme was dissolved in water containing 25mM-KH_2PO_4. pH values were re-adjusted to about 6.8 by addition of concentrated buffer (Mes or Mops), so that e.p.r. signals showing minimum or zero contamination of the high-pH or low-pH species would be obtained. Samples (final haem concentration about 100μM) in e.p.r. tubes were reduced aerobically by addition of sulphite (final concentration 1.0mM) and frozen within 3 min.

Preparation of ^{17}O-labelled phosphate

Preparation of ^{17}O-labelled phosphate was essentially that used for ^{18}O phosphate by Boyer & Bryan (1967). Potassium dihydrogen phosphate (2M) in 52·7atom% ^{17}O-enriched water (Monsanto Research Corporation, Miamisburg, OH 45342, U.S.A.) was heated in a sealed tube at 124°C for 7 days. The sample was then evaporated to dryness over a desiccant at 50°C and the enriched phosphate so obtained was redissolved in normal H_2O to a final concentration of 1M. This solution was diluted appropriately for use.

E.p.r. measurements

E.p.r. spectra were recorded at about 120K on a Varian E9 spectrometer at 9 or 35GHz. Modulation amplitudes at the two frequencies were 0.25 and 1.25mT, with microwave powers of 5mW and 10dB attenuation respectively. Computer-generated difference spectra were obtained as described previously (Bray *et al.*, 1978). Computer simulations of e.p.r. spectra employed the programs of Lowe (1978).

Results

Reduction of freeze-dried sulphite oxidase dissolved in normal H_2O with sulphite in the presence of phosphate ions and at moderately low pH produces the typical Mo(V) e.p.r. signal of the phosphate complex, as described by Lamy *et al.* (1980). Generation of the signal with freeze-dried enzyme dissolved in ^{17}O-enriched water gave essentially the same spectrum (Fig. 1*a*), as is illustrated by the featureless difference spectrum of Fig. 1(*b*). Incubation of the phosphate enzyme complex in ^{17}O-enriched water for periods of up to at least 3h before reduction did not change the spectrum appreciably. In contrast, reduction of sulphite oxidase in the presence of ^{17}O-labelled phosphate gave rise to changes in the signal (Fig. 1*c*) due to the interaction of the ^{17}O with molybdenum.

The spectrum of Fig. 1(*c*) must contain contributions from both the enriched and normal phosphate signals and subtraction of an appropriate amount of unenriched spectrum should give the spectrum of the ^{17}O-split species. The amount of unenriched spectrum subtracted was varied until features obviously due to it just disappeared in the difference spectrum. The final difference spectrum so obtained is illustrated in Fig. 1(*d*). Making the simplifying assumption (see the Discussion section) of a single oxygen atom coupled to molybdenum, then intensity of the ^{17}O spectrum (Fig. 1*d*) relative to that of the mixed spectrum (Fig. 1*c*) should correspond to the percentage enrichment of ^{17}O in

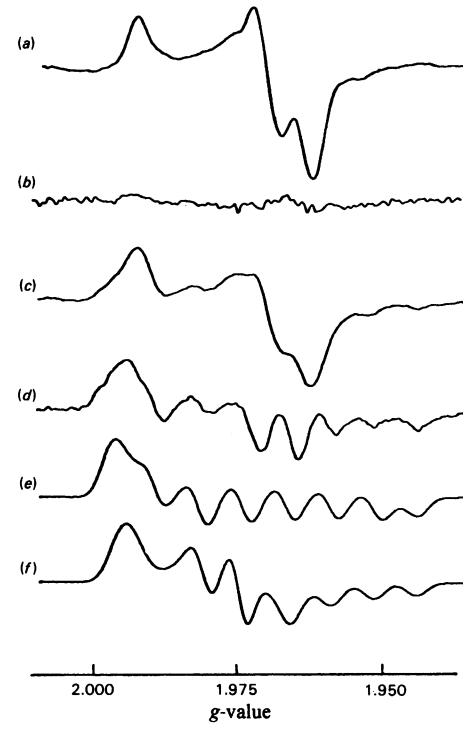

Fig. 1. *E.p.r. spectra of the phosphate complex of sulphite oxidase at 9.3 GHz*

(*a*) Shows the enzyme reduced with sulphite in 25mM-phosphate in ^{17}O-enriched H_2O, and (*b*) is a difference spectrum showing this signal minus a corresponding one obtained in normal water, illustrating the absence of ^{17}O splittings in (*a*). In (*c*) the phosphate spectrum has been obtained by reducing the enzyme in the presence of ^{17}O-labelled phosphate and the difference spectrum in (*d*) has been produced by subtracting appropriate amounts (see the text) of (*a*) from (*c*) and corresponds to $2 \times [(c) - 0.65(a)]$. The spectrum in (*e*) is a simulation of this difference spectrum by using as *g*-values: g_1, 1.9917; g_2, 1.9692 and g_3, 1.9614 (Lamy *et al.*, 1980) and ^{17}O hyperfine couplings of A_1, 0.10; A_2, 1.30 and A_3, 1.25mT. Half-linewidths were Δ_1, 0.33; Δ_2, 0.27 and Δ_3, 0.29mT. (*f*) Is an alternative simulation with hyperfine couplings of A_1, 0.10; A_2, 0.30 and A_3, 1.25mT.

the phosphate, that is, about 50% (allowing for incomplete conversion in the preparation of the phosphate). Integrations of the spectra were consistent with this, within experimental error. It was found, furthermore, that if the proportion subtracted was varied by more than ±10% from that indicated in Fig. 1(*d*), then improbable lineshapes, that is ones which could not obviously be simulated, resulted.

Fig. 1(*e*) is a simulation of the difference spectrum (Fig. 1*d*) obtained by using the parameters given in the legend to the Figure. The coupling of the oxygen to the molybdenum is clearly strong and anisotropic. Note that in the difference spectrum (Fig. 1*d*), the g_1 feature is apparently shifted to lower field by 0.4 mT in comparison with the unsplit spectrum (Fig. 1*a*). The simulation program (Lowe, 1978) reproduced this effect (Fig. 1*e*) and indicated that it was due to a low-field angular anomaly (Rollmann & Chan, 1969), presumably related to the highly anisotropic nature of the hyperfine coupling.

Another set of parameters with a small value of A_2 (Fig. 1*f*) also gave an acceptable simulation of the difference spectrum of Fig. 1(*d*). 35 GHz spectra were therefore obtained, not only to distinguish between the two sets of parameters, but also to decrease effects of the low-field angular anomaly (Rollman & Chan, 1969) and so to confirm the very small A_1 value. Figs. 2(*a*) and 2(*b*) are 35 GHz spectra of the unenriched phosphate complex and of the complex obtained with isotopically labelled phosphate respectively. The 35 GHz difference spectrum [equivalent of Fig. 1(*d*) and obtained similarly] is shown in Fig. 2(*c*). The simulation (Fig. 2*d*), calculated with parameters used for Fig. 1(*e*), shows good fitting both in the g_1 region confirming the small A_1 value and in the overlapping g_2 and g_3 regions. With the second set of parameters (Fig. 2*e*), which clearly must be rejected, the fit in the g_2, g_3 region is particularly poor.

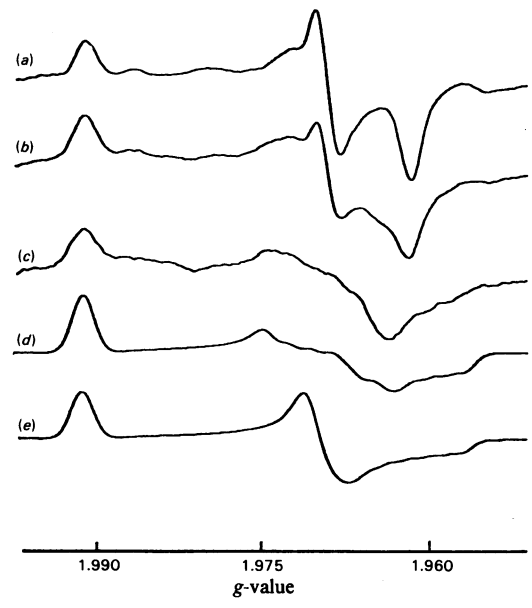

Fig. 2. *E.p.r. spectra of the phosphate complex of sulphite oxidase at 34.7 GHz*

The spectra shown in (*a*) and (*b*) are as in Figs. 1(*a*) and 1(*c*) respectively, except for having been recorded at the higher microwave frequency. The difference spectrum in (*c*) has been obtained by subtracting appropriate amounts (see the text) of (*a*) from (*b*) and corresponds to $2 \times [(c) - 0.6\,(a)]$. The simulations in (*d*) and (*e*) have been calculated with the parameters used for Figs. 1(*e*) and (*f*), with the addition of 0.25 mT to all half-linewidths. Clearly the simulation of (*d*) is preferred to that of (*e*). Conditions for generating the spectra were those given in the legend to Fig. 1.

Discussion

Oxyanion complexes of molybdenum-containing enzymes distinguishable by e.p.r. of molybdenum(V) include NO_3^- and NO_2^- complexes of nitrate reductase and NO_3^- and borate complexes of xanthine oxidase (Vincent & Bray, 1978; Gutteridge *et al.*, 1978*a*,*b*; Malthouse *et al.*, 1980; Gutteridge & Bray, 1980*b*). Phosphate is so far the only oxyanion that changes the e.p.r. signals of sulphite oxidase significantly (Lamy *et al.*, 1980). The phosphate signal, though derived from this enzyme under low-pH conditions (M. T. Lamy & S. Gutteridge, unpublished work), is unique among the anion complexes in exhibiting no proton hyperfine structure. Furthermore, the present results show that, in contrast with the low- and high-pH signals of sulphite oxidase, there is no interaction in the phosphate complex between ^{17}O from enriched water and the molybdenum.

The above facts, together with the strong coupling of the oxygen of phosphate to molybdenum, suggest strongly that, in the complex, the phosphate ion has become co-ordinated to molybdenum. In so doing an oxygen ligand must have been displaced, which, depending on pH and oxidation state of the metal, was either a hydroxy group or a terminal oxygen atom. The largest value for the oxygen hyperfine coupling ($A_3 = 1.3$ mT) is similar to ^{17}O couplings in xanthine oxidase (Gutteridge *et al.*, 1979; Gutteridge & Bray, 1980*a*). So large a splitting suggests that the oxygen of the phosphate is a ligand of the molybdenum, occupying an equatorial type of position in the no-doubt-distorted geometry of the co-ordination sphere.

According to available data on complexes of the pyrophosphate ion with molybdenum(V) (Imamura

et al., 1976), some interaction of the phosphorus nucleus ($I = \frac{1}{2}$) with molybdenum is to be expected. In keeping with this, linewidths of the normal phosphate signal are somewhat larger than those of the other Mo(V) signals of sulphate oxidase (Lamy *et al.*, 1980). Unresolved phosphorus splittings seem to be of the order of 0.1 mT, indicating rather weak coupling.

The amount of structural information concerning the molybdenum–phosphate complex at present derivable from e.p.r. is limited. The anisotropic nature of the coupling is indicative of a large *p*-orbital contribution to the bonding. Obviously, if there is more than one exchangeable oxygen atom bound to the molybdenum, then phosphate could be bound as a bidentate rather than a monodentate ligand. However, bearing in mind the reasonably satisfactory simulations which we have obtained, it seems that, at the present level of enrichment with ^{17}O and at the present signal-to-noise ratios of the spectra, a more sophisticated analysis of the data aimed at extracting coupling constants for two phosphate oxygen atoms is not justified. Unfortunately, although e.x.a.f.s. indicates the presence of two oxygen groups bound to molybdenum in oxidized sulphite oxidase (Cramer *et al.*, 1979*a*), the data of these workers was apparently collected from enzyme almost wholly in the high-pH form. Therefore, until higher enrichments of $H_2{}^{17}O$ become available to us or until further e.x.a.f.s. studies are performed on the other species of sulphite oxidase, a complete description of the formation of the phosphate inhibitor complex will not be forthcoming.

Our results provide new insights both into the mechanism of inhibition of sulphite oxidase by phosphate and into the nature of the enzymic reaction itself. Binding of phosphate and of sulphate, which are structurally analogous to one another, may both involve displacement of an oxygen ligand of the molybdenum by an oxygen of the anion. The leaving oxygen atom would presumably depart in the form of a hydroxyl ion (cf. Gutteridge & Bray, 1980*a*). Conversely, it might be postulated that binding of sulphite to the enzyme occurs without displacement of oxygen, allowing direct transfer of this atom from the molybdenum to yield a sulphate product complex (not detectable by e.p.r.) analogous to the phosphate complex. However, despite recent work with model compounds, also favouring an oxygen-transfer mechanism (Newton *et al.*, 1979), direct oxygen transfer has yet to be demonstrated for this enzyme and the part played by proton transfer in the reaction (cf. Bray, 1980) remains to be elucidated.

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