The nature of the phosphate complex of sulphite oxidase from electron-paramagnetic-resonance studies

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The phosphate complex of sulphite oxidase in the Mo(V) oxidation state was investigated by e.p.r. spectroscopy. Third-derivative spectra reveal a wealth of structural detail previously unobserved in this spectrum. Most notable is the presence of hyperfine coupling from two inequivalent $I = \frac{1}{2}$ nuclei, which we tentatively attribute to two ^{31}P nuclei. Unresolved hyperfine interactions from at least one exchangeable ^1H nucleus are also present.

INTRODUCTION

Sulphite oxidase is the molybdenum–haem protein that catalyses the physiologically important oxidation of sulphite to sulphate, a reaction that is thought to take place at the molybdenum site (Bray, 1980, 1988). Mo(V) e.p.r. spectroscopy has provided much important information relating to the structures of the active sites of the molybdenum enzymes (see Bray, 1980, 1988). Early work (Kessler & Rajagopalan, 1974) showed that phosphate is an effective inhibitor of sulphite oxidase, and forms a characteristic Mo(V) e.p.r. signal known as the phosphate signal (Lamy et al., 1980). The phosphate signal has been quite extensively characterized at both 9 GHz and 35 GHz microwave frequencies (Lamy et al., 1980; Gutteridge et al., 1980). Furthermore, Gutteridge et al. (1980) have used ^{17}O-enriched phosphate; they detected only a single ^{17}O coupling, but nevertheless concluded that phosphate was probably attached as a bidentate ligand with the second co-ordinated oxygen atom giving unresolved ^{17}O hyperfine coupling. We present herein a further characterization of the sulphite oxidase phosphate Mo(V) e.p.r. signal, and demonstrate the presence of hyperfine coupling from exchangeable protons and from two different $I = \frac{1}{2}$ nuclei, which are most probably ^{31}P of bound phosphate groups.

MATERIALS AND METHODS

Sulphite oxidase was purified from chicken liver by a modification of the procedure of Kessler & Rajagopalan (1972) (C. A. Kipke, R. A. Sunde & J. H. Enemark, unpublished work). Enzyme samples at a concentration of approx. 0.1 mM-Mo (in 50 mM-Pipes buffer in the presence of 20 mM-K_2HPO_4, at pH 7.0) were anaerobically reduced with 0.5 mM-sulphite and frozen in 3 mm-internal-diameter quartz tubes. E.p.r. spectra were recorded with a Varian E109 instrument interfaced to an ACT Apricot Xi computer, and equipped with an Oxford Instruments ESR 900 liquid-helium flow cryostat and an EIP 548A microwave frequency counter. Typical spectrometer conditions were 0.1 mT modulation amplitude, 5 mW applied microwave power and a temperature of 95 K. Mn^{2+}–diphenylpicrylhydrazyl was used as a reference for spectrometer calibrations. Spectra were transferred to a Digital Equipment Corporation VAX 11-8350 computer for manipulation and simulations, the latter performed by using a second-order perturbation solution approach (George & Bray, 1988). The spectra were fitted by using a modified Levenberg–Marquardt algorithm as described by George & Bray (1988). Hyperfine couplings to ^2H ($I = 1$) were calculated from the values for ^1H and the ratio of nuclear g-values (0.1535; see Goodman & Raynor, 1970).

RESULTS AND DISCUSSION

The first-derivative Mo(V) e.p.r. spectrum of the phosphate complex is shown in Fig. 1(a); it is very similar to that reported earlier by Bray and co-workers (Lamy et al., 1980). These workers interpreted the spectrum as a simple rhombic signal showing no resolved hyperfine structure (Lamy et al., 1980), and also reported that the signal did not change upon exchange into ^2H_2O, indicating a lack of exchangeable protons at the molybdenum site. Gutteridge et al. (1980), however, noted that the e.p.r. linewidths used in the computer simulation of the signal were somewhat larger than those of the other Mo(V) e.p.r. signals from sulphite oxidase, and suggested that this might be due to unresolved hyperfine coupling from the ^{31}P nucleus of phosphate. The presence of such additional structure in the phosphate signal is shown definitively in the third-derivative e.p.r. spectrum (Fig. 1c). The g_y region of the third-derivative lineshape is appreciably sharpened for samples prepared in ^2H_2O (Fig. 1d), indicating the presence of unresolved hyperfine structure from at least one exchangeable proton, which might indicate the presence of Mo–OH ligation (e.g. Bray, 1988). It is not particularly surprising that the additional structure of the phosphate signal described here was missed in the earlier work, as the effects in the usual first-derivative lineshape (Figs. 1a and 1b) are extremely subtle. This, together with the slightly higher modulation amplitudes used earlier, conspired to hide the effects of the additional structure.

Computer simulation of the spectra indicates, in addition to the unresolved coupling from ^1H, the presence of

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hyperfine coupling from two inequivalent $I = \frac{1}{2}$ nuclei. The couplings from these nuclei are approximately equivalent in the g_y direction, yielding a $1:2:1$ triplet structure centred at about $g = 1.97$, but are inequivalent in the g_x direction, yielding a doublet structure centred at about $g = 1.96$, and are unresolved in the g_z direction. An alternative explanation, that the phosphate signal arises
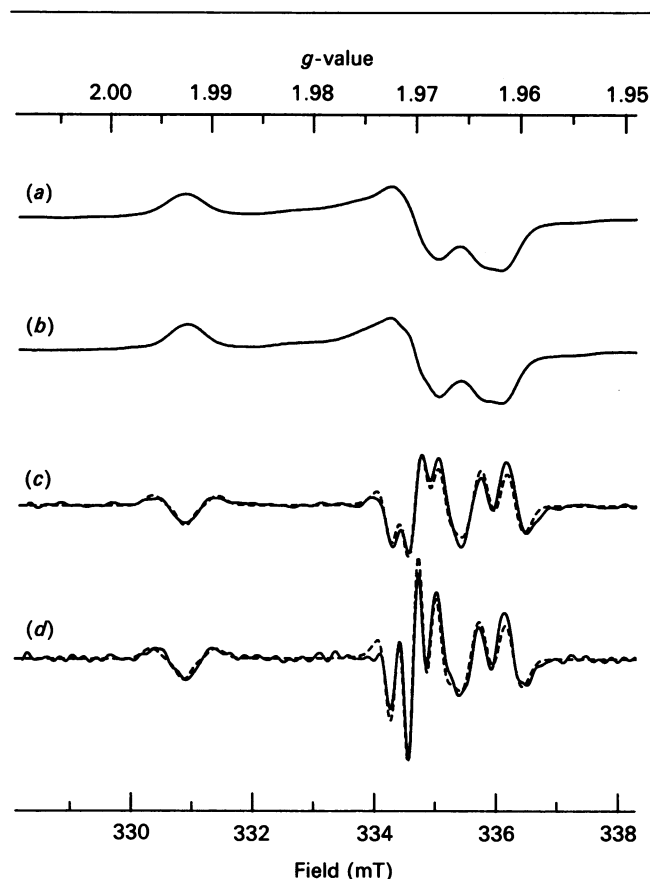

Fig. 1. Mo(V) e.p.r. spectra of the phosphate complex of sulphite oxidase

(a) and (b) are first-derivative spectra of enzyme in $^{1}H_{2}O$ and in $^{2}H_{2}O$ buffers respectively. (c) and (d) are the corresponding third-derivative spectra. The continuous lines are experimental traces, and the dashed lines show simulations calculated by using the parameters in Table 1. Traces (c) and (d) have been smoothed by using Fourier filtering techniques to the resolution defined by the modulation amplitude (0.01 mT).

from two equally abundant species, both coupled to a single $I = \frac{1}{2}$ nucleus with similar values for g_x and g_z, but with g_y values differing so as to give the appearance of a $1:2:1$ triplet, can be dismissed, as earlier Q-band work (Lamy et al., 1980; Gutteridge et al., 1980) would have resolved the two signals.

Unambiguous identification of the two $I = \frac{1}{2}$ nuclei, provisionally assigned as ^{31}P, is not possible from e.p.r. data alone (there are no stable magnetic isotopes of phosphorus other than the 100% naturally abundant ^{31}P). Nevertheless, the number of potential candidates is actually quite small, and, excluding such unlikely possibilities as ^{19}F, the only feasible possibilities are ^{31}P and ^{1}H. Protons seem less likely, both because the other e.p.r. signals from the enzyme show no similar structure (from a non-exchangeable proton), and because no particularly intense proton spin-flip transitions (George, 1985), which might be expected at orientations where the coupling is weaker (e.g. along g_x or g_z), were observed. The observed hyperfine structure most probably originates from two inequivalently coupled ^{31}P nuclei, implying that two phosphate groups, rather than one, bind to the molybdenum in the inhibited complex. An alternative interpretation is that one of the ^{31}P hyperfine couplings originates from the phosphate group of the molybdenum cofactor (see, e.g., Johnson et al., 1984), but the absence of any similar couplings in the other Mo(V) e.p.r. signals of the enzyme makes this seem rather unlikely.

A phosphate ligand of molybdenum would be co-ordinated via its oxygen atoms(s) in a $Mo–O–P(O_3)$ arrangement. Bearing this in mind, our interpretation of two bound phosphate groups seems, at first sight, to be contradicted by the results obtained by Gutteridge et al. (1980), who detected the presence of only a single coupled oxygen atom for the phosphate signal developed with ^{17}O-enriched phosphate. For d^1 systems such as Mo(V), however, hyperfine couplings to ligands directly co-ordinated to the metal (i.e. ^{17}O for a Mo–O–P arrangement) are very often weaker than to atoms adjacent to these ligands (i.e. ^{31}P). Although the exact mechanism for this phenomenon is obscure, a qualitative explanation (see George & Bray, 1983, and references cited therein) is that the atom directly co-ordinated to molybdenum (i.e. ^{17}O) will lie on a node of the half-filled ground-state orbital, resulting in a small hyperfine coupling, while the adjacent atom (i.e. ^{31}P) might be held by the non-linear Mo–O–P bond into a position where it can overlap with the ground-state orbital, yielding a large hyperfine coupling. For the sulphite oxidase–phosphate complex, this

Table 1. Parameters of the sulphite oxidase phosphate Mo(V) e.p.r. signal

g-values are considered to be accurate to ± 0.0005; the values presented here differ slightly from those reported by Gutteridge et al. (1980), although the differences are within the range of errors specified. Values for hyperfine couplings are in MHz ($1\,mT = 27.6\,MHz$ at $g = 1.97$). Values for half-linewidths are in mT.

	x	y	z	Average
g	1.9625	1.9701	1.9926	1.9751
A (^{31}P)	10.4	7.2	8.3*	8.6
A (^{31}P)	4.1	7.2	8.3*	6.5
A (^{1}H)	0.8*	3.3	0.8*	1.6
$\triangle$	0.19	0.14	0.21	

* The value for this parameter is approximate because the splitting is not resolved in the e.p.r. spectrum.

phenomenon, together with the smaller inherent couplings of ^{17}O than ^{31}P (Goodman & Raynor, 1970), might render an oxygen ligand undetectable while still allowing the detection of the ^{31}P.

Our results may have some bearing on the catalytic mechanism of sulphite oxidase. Bray and co-workers (Bray et al., 1983; Bray, 1986) have proposed that sulphite binds to molybdenum as a bidentate ligand during the catalytic cycle. This would require two vacant co-ordination sites, which could be occupied by two monodentate phosphate ligands in the phosphate-inhibited complex.

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