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Evidence from electron-paramagnetic-resonance spectroscopy for a complex of sulphite ions with the molybdenum centre of sulphite oxidase

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Reduction of sulphite oxidase by sulphite at low pH values in Mes (4-morpholine-ethanesulphonic acid) buffer gives rise to a new molybdenum(V) electron-paramagnetic-resonance spectrum different from that obtained by photoreduction of the enzyme in the same medium. The spectrum is attributed to a sulphite complex of the enzyme, showing *g*-values of about 2.000, 1.972 and 1.963. The complex is analogous to that with the inhibitor phosphate in that it gives rise to no observable hyperfine coupling of Mo(V) to exchangeable protons.

Studies of the structures of complexes of enzymes with substrates or inhibitors are particularly important in the elucidation of enzymic mechanisms. In the case of molybdenum-containing enzymes, much structural information concerning their molybdenum centres is available from e.p.r. spectroscopy of Mo(V). Xanthine oxidase shows well-defined spectra from complexes of Mo(V) with purine molecules. Some of these may represent true Michaelis complexes of this enzyme (Bray, 1980). For sulphite oxidase, although we have studied the spectrum of the complex of the enzyme with the inhibitor, inorganic phosphate (Lamy *et al.*, 1980; Gutteridge *et al.*, 1980), no Mo(V) e.p.r. spectrum from a substrate complex has so far been described. We now present evidence for such a complex and discuss its structure in relation to the catalytic mechanism of the enzyme.

Materials and methods

Sulphite oxidase samples were prepared and e.p.r. spectra recorded and manipulated as we described previously (Lamy *et al.*, 1980). E.p.r. conditions were generally about 120 K, 9.3 GHz, 20 mW power and 0.2 mT modulation amplitude. pH measurements refer to those obtained with a micro-electrode after the samples had been thawed after recording their spectra. Apparent pH values in ^2H_2O refer to uncorrected readings from a meter standardized in ordinary water. Photoreduction was carried out in the presence of 5 μM-10-methyl-5-deazaflavin 3-propanesulphonate (kindly provided by Professor P. Hemmerich, University of Konstanz, Konstanz, Germany) and 5 mM-EDTA.

Results and discussion

We found previously (Lamy *et al.*, 1980) that sulphite was not essential for generating Mo(V) spectra from sulphite oxidase and that, for example, photoreduction of the enzyme in the presence of a deazaflavin derivative (Massey & Hemmerich, 1977) was also effective. Under the conditions we employed previously (Lamy *et al.*, 1980) there was little difference between the spectra of sulphite- and photo-reduced enzyme. We now find, however, that this is not the case under all conditions. Thus, with enzyme in dilute Mes (4-morpholine-ethanesulphonic acid) buffer, pH 6.5–6.6, the spectrum of Fig. 1(*a*) was obtained for reduction by sulphite and that of Fig. 1(*b*) for photoreduction. Clearly there are substantial differences.

We reported previously (Lamy *et al.*, 1980) that spectra from sulphite oxidase generated under most conditions were a mixture of those from a high-pH form and a low-pH form. Spectra of these workers for these enzyme forms are replotted respectively in Figs. 1(*d*) and 1(*g*). Comparison of Fig. 1(*d*) with Figs. 1(*a*) and 1(*b*) indicates that there are contributions from the high-pH form in both. When the high-pH form was subtracted out, by using the spectral difference techniques of Bray *et al.* (1978), the spectrum of Fig. 1(*e*) was obtained for the sulphite-reduced enzyme and that of Fig. 1(*f*) for the photoreduced sample. The latter spectrum is very similar to, if not identical with, the standard low-pH spectrum of Fig. 1(*g*) (unpublished work by

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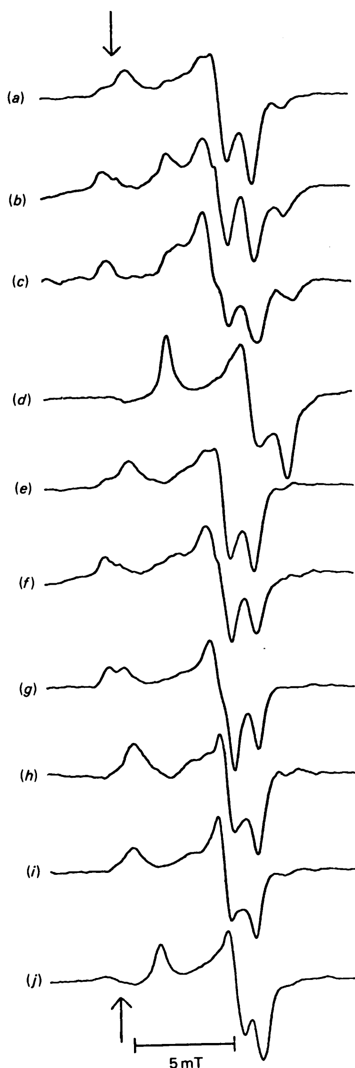

Fig. 1. *E.p.r. spectra of Mo(V) in sulphite oxidase*
(*a*)–(*c*) and (*i*) are experimental spectra obtained by reducing the enzyme in Mes buffer (20 mM), pH 6.2–6.6. All samples were in ordinary water, except (*i*), which was in ^2H_2O (approx. 90%). In (*a*) and (*i*), sulphite (5 mM) was used as reductant. In (*b*) and (*c*), photoreduction in the presence of a deazaflavin derivative was employed (see the Materials and methods section). In (*c*), sodium sulphate (10 mM) was also present. (*e*), (*f*) and (*h*) are difference spectra obtained from (*a*) and (*b*) as indicated below. (*d*), (*g*) and (*j*) are experimental or difference spectra replotted and re-scaled from our previous work (Lamy *et al.*, 1980); (*d*) is the 'high-pH' spectrum, (*g*) is the 'low-pH (Cl^-)' spectrum, and (*j*) is the 'phosphate' spectrum. (*e*) was obtained by subtracting (*d*) from (*a*) and scaling the spectrum appropriately and represents the spectrum obtained in the presence of sulphite after removal of the high-pH component. Similarly, (*f*) was obtained by subtracting (*d*) (and a small amount of a blank signal) from (*b*), and thus represents the pure, photoreduced, low-pH spectrum. Finally, (*h*) was obtained by subtracting (*f*) from (*e*) and corresponds to the spectrum attributed to the sulphite complex; a similar spectrum was obtained directly in (*i*). Final pH values were as follows: (*a*) 6.5, (*b*) 6.6, (*c*) 6.2, (*i*) 6.3. The arrow corresponds to $g =$ 2.0037.

S. Gutteridge, M. T. Lamy and R. C. Bray suggests that this species may represent a chloride complex of the low-pH form of the enzyme). [Note that the characteristic g_1 doublet of the low-pH form, which is very close to the free-radical region of the spectrum, appears to be partly obscured in Fig. 1(*f*), possibly by a contaminating radical.] In contrast with Fig. 1(*f*), Fig. 1(*e*) (the corrected spectrum for the sulphite-reduced sample) appears to represent a mixture of species. There appears to be a contribution from the low-pH form, and on subtracting Fig. 1(*f*) from Fig. 1(*e*) (so as to eliminate the feature at the lowest field value) the spectrum of Fig. 1(*h*) was obtained.

The possibility that Fig. 1(*h*) corresponds to the spectrum of a sulphite complex of the reduced enzyme immediately arises. The spectrum is analogous to that from the phosphate complex of the enzyme [described by us previously (Lamy *et al.*, 1980) and replotted in Fig. 1(*j*)] in that it appears to be a simple rhombic form showing no hyperfine coupling to protons. Striking confirmation of this assignment is provided by the spectrum illustrated in Fig. 1(*i*). The sample was prepared at a slightly lower pH value than were the other samples, in order to avoid the necessity for subtracting the high-pH form, and in ^2H_2O solution, in order to check on the absence of proton splittings. In a parallel experiment (not illustrated), in which a similar sample in ^2H_2O was photoreduced, the main contribution to the spectrum was from the ^2H form (Lamy *et al.*, 1980) of the low-pH spectrum. However, this form appears to make relatively little contribution to the spectrum of the sulphite-reduced sample in Fig. 1(*i*). Thus Fig. 1(*i*) represents, we believe, the almost pure spectrum of the sulphite complex of the enzyme. Parameters measured from this spectrum are as follows: g_1 1.9996, g_2 1.9720, g_3 1.9629, $g_{av.}$ 1.9782.

It was important to establish the point, which we have not considered so far, that the spectrum of Fig. 1(*i*) is due to a sulphite complex and not to a sulphate complex. Fig. 1(*c*) illustrates an experiment carried out parallel to those of Figs. 1(*a*) and 1(*b*), in which the enzyme was photoreduced in the presence of sulphate. Though the spectra of samples photoreduced in the presence (Fig. 1*c*) and absence (Fig. 1*b*) of sulphate are not identical, and further work is required to characterize the difference, it is clear,

nevertheless, that the very characteristic g_1 feature of the spectrum of Fig. 1(*i*) is absent from Fig. 1(*c*). Therefore we attribute Fig. 1(*i*) to a sulphite, rather than to a sulphate, complex.

Formation of a spectroscopically detectable complex between the substrate, sulphite, and the enzyme sulphite oxidase calls for comment. The redox potential for the system sulphite/sulphate is much lower than that for molybdenum in the enzyme (Cramer *et al.*, 1980). Therefore a Michaelis complex of the oxidized enzyme with the substrate would be expected to transform itself rapidly to a sulphate product complex. In so doing, two electrons would be transferred from the substrate to molybdenum, with one of the electrons subsequently moving, in at least some of the molecules, on to the haem group of the enzyme, thus leaving molybdenum in the Mo(V) state. After dissociation of the product, the enzyme could then form a Michaelis-like complex with another sulphite molecule. However, in this oxidation state, molybdenum would be unable to accept two further electrons from the substrate so that this complex, observable by e.p.r., is a dead-end one. Further catalysis in such molecules would not occur until the haem was itself reoxidized, enabling it to reoxidize molybdenum to Mo(V).

We suggested previously (Gutteridge *et al.*, 1980) that phosphate forms a complex of the enzyme in which an oxygen ligand of molydenum (terminal oxygen in the oxidized enzyme or –OH in the reduced enzyme) has been replaced by one of the oxygen atoms of the phoshate group. This labile oxygen atom of the enzyme was the one presumed to be available for transfer to sulphite, when this is bound to the active centre. The lack of coupled protons in the phosphate and sulphite complexes of the reduced enzyme argues for structural analogies between them. Since the phosphate complex is apparently more stable, and is thus likely to be more amenable to experimental study than is the sulphite complex, further work on its structure, for example by X-ray spectroscopy, will be of particular interest.

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