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Studies by electron-paramagnetic-resonance spectroscopy of the molybdenum centre of aldehyde oxidase

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Molybdenum(V) e.p.r. spectra from reduced forms of aldehyde oxidase were obtained and compared with those from xanthine oxidase. Inhibited and Desulpho Inhibited signals from aldehyde oxidase were fully characterized, and parameters were obtained with the help of computer simulations. These differ slightly but significantly from the corresponding parameters for the xanthine oxidase signals. Rapid type 1 and type 2 and Slow signals were obtained from aldehyde oxidase, but were not fully characterized. From the general similarities of the signals from the two enzymes, it is concluded that the ligands of molybdenum must be identical and that the overall co-ordination geometries must be closely similar in the enzymes. The striking differences in substrate specificity must relate primarily to structural differences in a part of the active centre concerned with substrate binding and not involving the catalytically important molybdenum site.

Aldehyde oxidase is one of the group of enzymes referred to as molybdenum-containing hydroxylases (Bray, 1975). Though it has many properties in common with xanthine oxidase, the specificity patterns of the two enzymes are substantially different (Krenitsky *et al.*, 1972). Substrates reduce these enzymes to forms containing Mo(IV) and Mo(V), and studies by e.p.r. spectroscopy of the metal in the latter oxidation state have provided extensive information on the structures and mechanisms of action of the molybdenum centres.

The greater part of such information (Bray, 1980*a*) relates to milk xanthine oxidase. However, the centres in xanthine dehydrogenases from avian (Barber *et al.*, 1976) or from bacterial (Dalton *et al.*, 1976) sources differ little from those in the milk enzyme. A number of distinct Mo(V) e.p.r. signals from these enzymes have been characterized and given names (Bray, 1980*a*). Each signal is obtainable under specific conditions and corresponds to a different configuration of the molybdenum centre. Signals from the functional enzymes include Rapid types 1 and 2. An inhibitory side reaction with aldehyde substrates (Malthouse *et al.*, 1981*a*) (or with certain alcohols) gives rise to the Inhibited signal. The enzymes are susceptible to inactivation by the loss of a sulphur ligand from the molybdenum centre to give the desulpho forms (Bray, 1975). These in turn give rise (Bray, 1980*a*) to the Slow signal and to the Desulpho Inhibited signal (originally named Resting II).

Though it has been established (Branzoli & Massey, 1974*b*) that aldehyde oxidase can be converted into a desulpho form, nevertheless studies on Mo(V) in the enzyme by e.p.r. (Rajagopalan *et al.*, 1968*a*,*b*) are at a much less advanced state than are those on other enzymes of this group. The aim of the present work was, by using e.p.r. of Mo(V), to compare the molybdenum centre of aldehyde oxidase with that in the other molybdenum-containing hydroxylases.

Materials and methods

Enzymes

Partially purified aldehyde oxidase was prepared from frozen rabbit livers obtained from a commercial rabbit farm. The method of Branzoli & Massey (1974*a*) was followed as far as the acetone fractionation step. Although the enzyme at this stage is relatively crude, contamination by other molybdenum-containing hydroxylases was not a problem. Their content in rabbit liver is low (Krenitsky *et al.*, 1974), and we were unable to detect xanthine oxidase activity in our samples. The enzyme was concentrated by ultrafiltration (Minicon B15 concentrator from Amicon, Woking,

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Surrey, U.K.). Milk xanthine oxidase was prepared by the salicylate denaturation method of Hart *et al.* (1970).

E.p.r. samples and spectra

Enzyme samples in appropriate buffers contained in 4mm-internal-diameter e.p.r. tubes were reduced with substrates or dithionite and frozen. Spectra were recorded on a Varian E9 spectrometer linked to a computer and visual display system (Bray *et al.*, 1978). Recording conditions were as follows: temperature, about 120K; microwave frequency, 9.3GHz; power, 20mW; modulation amplitude, 0.16–0.25mT. Computer simulation of e.p.r. spectra used the program of Lowe (1978) or an iterative version of this (Malthouse *et al.*, 1981*b*). The *g* values are considered to be accurate to ±0.0002.

Results and discussion

Mo(V) e.p.r. spectra from inhibitory side reactions and from non-functional forms of aldehyde oxidase

Early e.p.r. work on aldehyde oxidase (Rajagopalan *et al.*, 1968*a*,*b*), though revealing complex spectra that were undoubtedly due to Mo(V), gave little indication of any signals arising from single chemical species. Since, however, treatment of the enzyme with methanol gave (Rajagopalan *et al.*, 1968*a*) a relatively well-defined signal that appeared at least in part (Bray & Swann, 1972; Bray, 1980*b*) to be analogous to the Inhibited signal (Pick *et al.*, 1971), we first looked for this signal from our preparations. We found that the Inhibited signal could be obtained essentially free from other signals, on treating our partially purified aldehyde oxidase with high concentrations of formaldehyde (Fig. 1*a*). A computer simulation of the signal is shown in Fig. 1(*a'*). Parameters used are given in Table 1 and are discussed below.

The Slow signal from desulpho aldehyde oxidase was obtained by reduction with dithionite and is illustrated in Fig. 1(*c*). We have not attempted to simulate this signal, and its appearance seems to be intermediate between that of the xanthine oxidase Slow signal, as obtained in Bicine [*NN*-bis-(2-hydroxyethyl)glycine] or other buffers, and that obtained in the presence of added nitrate ions (Gutteridge *et al.*, 1978).

Unlike the preparations obtained by Rajagopalan *et al.* (1968*a*,*b*), our untreated enzyme samples showed no well-defined 'resting' signals. Their absence from our samples indicates a lack of contamination by the relevant non-functional enzyme form (Bray, 1980*a*). By using the procedure of Lowe *et al.* (1976) and treating the sample used for Fig. 1(*c*) with ethylene glycol, we obtained the Desulpho Inhibited signal, as illustrated in Fig. 1(*b*). Since this signal was particularly well-defined, we performed the computer simulation shown in Fig. 1(*b'*) (see Table 1 for parameters).

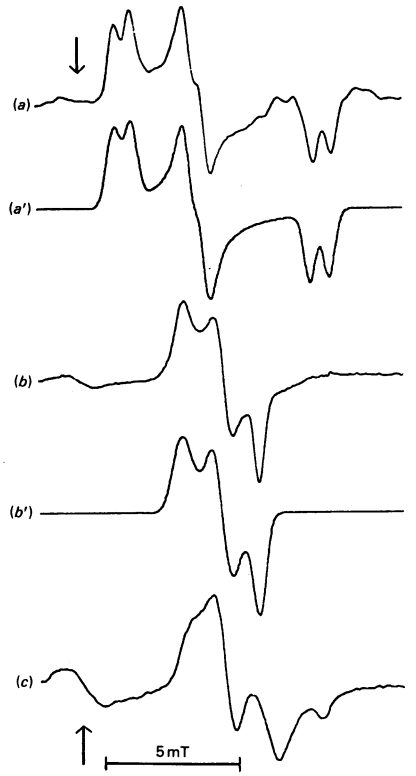

Fig. 1. *Mo(V) e.p.r. spectra from inhibitory side reactions and from non-functional forms of aldehyde oxidase*

Spectra (*a*) and (*a'*) show the Inhibited signal, spectra (*b*) and (*b'*) show the Desulpho Inhibited signal and spectrum (*c*) shows the Slow signal. Spectra (*a*), (*b*) and (*c*) are experimental spectra. Spectra (*a'*) and (*b'*) are computer simulations of spectra (*a*) and (*b*) respectively, with the use of the parameters given in Table 1. Here and in Fig. 2 the arrow corresponds to $g = 2.0037$. Reaction conditions for (*a*) were 60min with formaldehyde (0.2M) in 0.3M-Bicine/NaOH buffer, pH8.2, for (*c*) conditions were 20min with dithionite (5mM) in 10mM-Bicine buffer as above, and for (*b*) the sample used for (*c*) was thawed anaerobically, ethylene glycol was added to a concentration of 9M and the sample was refrozen after a further reaction period of 2h.

Spectra from functional aldehyde oxidase

The similarity of the spectra described above to the corresponding ones from milk xanthine oxidase encouraged us to examine spectra from aldehyde oxidase in the functional state. Brief reduction by most substrates would be expected, by analogy with xanthine oxidase, to give Rapid type 1 or type 2

Table 1. *Comparison of e.p.r. parameters for Mo(V) signals from aldehyde oxidase and xanthine oxidase*
Data for xanthine oxidase are taken from the literature (Bray, 1980*b*); those for aldehyde oxidase are the ones used for the simulations of Fig. 1. Hyperfine couplings and half-linewidths are in mT.

		g values (linewidth)				Hyperfine coupling [A(^{1}H)]			
Enzyme	Signal	1	2	3	av.	1	2	3	av.
Aldehyde oxidase	Inhibited (HCHO)	1.9926 (0.25)	1.9764 (0.22)	1.9501 (0.21)	1.9730 (0.23)	0.65	0.52	0.70	0.62
Xanthine oxidase	Inhibited (HCHO)	1.9911 (0.20)	1.9772 (0.14)	1.9513 (0.17)	1.9732 (0.17)	0.50	0.34	0.54	0.46
Aldehyde oxidase	Desulpho Inhibited (glycol)	1.9811 (0.30)	1.9724 (0.26)	1.9643 (0.22)	1.9726 (0.26)	—	—	—	—
Xanthine oxidase	Desulpho Inhibited (glycol)	1.9784 (0.27)	1.9707 (0.25)	1.9641 (0.17)	1.9710 (0.23)	—	—	—	—

spectra (see Figs. 2*b* and 2*f*). Both of these show splittings by exchangeable protons (Bray, 1980*a*). Type 1 spectra are nearly axial in form and are split into well-defined doublets by interaction with one strongly coupled proton; type 2 spectra are more rhombic, showing overlapping features with 1:2:1 intensity proportions from coupling to two nearly equivalent protons. Rajagopalan *et al.* (1968*a*,*b*) reported spectra, obtained on reducing aldehyde oxidase with substrates, which were only moderately like the xanthine oxidase Rapid signals. No evidence for proton splittings was deducible from the published spectra, which generally lacked sharp features.

Fig. 2(*a*) shows the spectrum obtained on reducing a sample of aldehyde oxidase with the substrate phthalazine (Stubley *et al.*, 1979). We obtained very similar spectra if other substrates, such as salicylaldehyde or purine, were substituted, and Fig. 2(*a*) is similar to the spectra reported for substrate-reduced aldehyde oxidase by Rajagopalan *et al.* (1968*a*). It shows no more than a general resemblance to the typical Rapid type 1 signal (Fig. 2*b*) (which we found to be obtainable on reducing xanthine oxidase with phthalazine).

A rather different spectrum from that of Fig. 2(*a*) was obtained (Fig. 2*c*) by pretreating aldehyde oxidase with the potential inhibitor 2-methylquinoline, then reducing it with phthalazine. [Stubley (1978) reported that 2-methylquinoline is oxidized by the enzyme to an inhibitor, thought to be 2-hydroxymethylquinoline.] Comparison of Figs. 2(*a*) and 2(*c*) suggested that substrate-induced spectra were probably mixtures of Rapid type 1 and type 2 signals. In confirmation of this, by using difference spectral techniques (Bray *et al.*, 1978) we were able to resolve these two spectra into a Rapid type 1 component (Fig. 2*d*) and a Rapid type 2 component (Fig. 2*e*). The general resemblance between Rapid type 1 spectra from the two enzymes is clear (compare particularly the split $g_\perp$ features in Figs. 2*b* and 2*d*). Though the spectrum of Fig. 2(*e*) is rather 'noisy', it does seem to have the typical type 2 form (cf. Fig. 2*f*).

In an attempt to obtain experimentally a Rapid type 2 spectrum uncontaminated with the type 1 form, we used reduction of the enzyme by dithionite in the presence of borate ions (Malthouse *et al.*, 1980). Spectra from aldehyde oxidase and from xanthine oxidase obtained under these conditions are shown in Figs. 2(*g*) and 2(*f*) respectively. Though a type 2 contribution to the aldehyde oxidase spectrum is indicated, particularly by the shoulders at the high-field end of the spectrum, a type 1 component is almost certainly present also (cf. Fig. 2*a*). The spectrum was not considered to be of sufficient quality to justify difference work, and computer simulation of the Rapid signals was not attempted.

Finally, to confirm our interpretations of the analogy between the aldehyde oxidase signals and the xanthine oxidase Rapid signals, we tried generating signals from aldehyde oxidase in ^2H_2O solution. The spectrum of Fig. 2(*h*) corresponds to aldehyde oxidase in ^2H_2O, reduced in the presence of borate, as in Fig. 2(*g*). Doublet features have entirely disappeared in this solvent and there has been a general narrowing of the spectrum. The changes demonstrate clearly the presence of coupled exchangeable protons in the signals from aldehyde oxidase.

Conclusions

Similarities between the Mo(V) spectra of aldehyde oxidase and those of xanthine oxidase, not obvious in earlier work (Rajagopalan *et al.*, 1968*a*,*b*), are now clear. For aldehyde oxidase, in seeking to derive structural information on the molybdenum centre, either functional forms of the enzyme or non-functional forms derived from them may with equal validity be compared with the

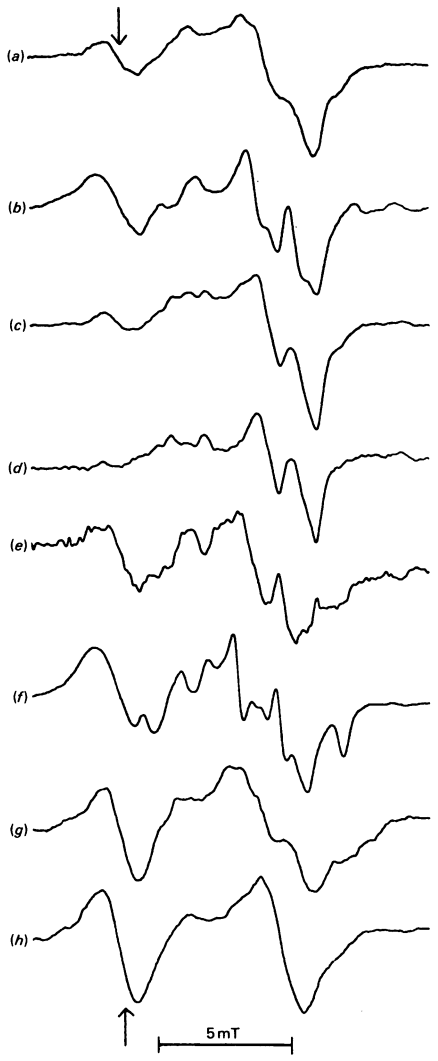

Fig. 2. *Mo(V) e.p.r. spectra from functional aldehyde oxidase: comparison with xanthine oxidase*

All spectra are of the Rapid type accompanied by various amounts of the flavin free radical signal. Spectra (*a*), (*c*), (*g*) and (*h*) are experimental spectra from aldehyde oxidase, spectra (*d*) and (*e*) are difference spectra derived from them, and spectra (*b*) and (*f*) are experimental spectra from xanthine oxidase. Spectra (*a*) and (*b*) were obtained by reduction for 1–2 min with phthalazine (5 mM). Spectrum (*c*) was similarly obtained after pre-incubation of aldehyde oxidase with 2-methylquinoline (40 mM) for 30 min. For spectra (*f*) and (*g*) reduction for 0.5 min with dithionite (1 mM) in the presence of 70 mM-sodium borate buffer, pH 8.2, was employed; spectrum (*h*) was obtained similarly to spectrum (*g*) except that the sample was in ^2H_2O (approx. 95% purity) in place of ordinary water. Spectrum (*d*) is a difference spectrum obtained by subtracting 0.6 of spectrum (*a*) from spectrum (*c*) and multiplying the resultant spectrum by 1.6. Similarly, spectrum (*e*) was obtained by subtracting 0.75 of spectrum (*c*) from spectrum (*a*) and multiplying by 3. Bicine/NaOH buffer, pH 8.2, was present in all samples at concentrations of 0.15 M for (*a*), 0.4 M for (*c*) and 10 mM for (*b*), (*f*), (*g*) and (*h*).

corresponding forms of xanthine oxidase. Computer simulation is obligatory for full evaluation of e.p.r. spectra of the type involved in the present work. We have simulated only two of the Mo(V) e.p.r. signals from aldehyde oxidase, namely Inhibited, which is derived from the active enzyme, and Desulpho Inhibited, from the inactive form lacking a sulphur ligand of molybdenum. Parameters for these (Table 1) differ slightly but quite significantly from those of the corresponding signals from xanthine oxidase (and xanthine dehydrogenases). The largest differences relate to coupling of the non-exchangeable proton of the Inhibited signal, increased by 30–40% in aldehyde oxidase, and the g_1 value in the Desulpho-Inhibited signal, increased by nearly 0.003.

For the other signals we investigated, namely Rapid types 1 and 2 and Slow, though full evaluation was not achieved, nevertheless it is clear that the aldehyde oxidase spectra are quite like the corresponding spectra from the other molybdenum-containing hydroxylases. The presence of exchangeable protons is particularly significant in this regard. In further work, it will be necessary to make use of spectra recorded at 35 GHz and to do further studies in ^2H_2O. We have not tried to obtain the xanthine Very Rapid signal, since this is not a substrate of aldehyde oxidase (Krenitsky *et al.*, 1972).

The fact that aldehyde oxidase yields Mo(V) e.p.r. signals clearly belonging to the same families as, and (where these have been established) having only slightly different parameters from, the other molybdenum-containing hydroxylases leads us to conclude that the environments of molybdenum in all these enzymes are essentially identical. For those species we studied in detail (Table 1), and presumably by analogy with these for the other species also, ligands of molybdenum and overall co-ordination geometries must be the same, with minor changes in bond angles or distances or in electron delocalization accounting for the small variations in parameters. The striking difference in substrate specificity that exists between aldehyde oxidase and the other enzymes must therefore be due primarily to structural differences in regions of the active centres concerned solely with substrate binding, rather than to differences in the catalytically important molybdenum sites. For xanthine oxidase, relative yields of the Rapid type 1 and type 2 signals under differing conditions have not been fully documented, but they appear to depend on equilibria between the signal-

giving species, governed by pH, the nature of the buffer and the presence of substrate and product molecules (R. C. Bray, S. Gutteridge & J. P. G. Malthouse, unpublished work). It seems that for aldehyde oxidase equilibrium constants in these transformations must differ significantly from those for xanthine oxidase. The significance of this in relation to the catalytic processes is at present uncertain, however.

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