The nature of the sulphur atom liberated from xanthine oxidase by cyanide

Evidence from e.p.r. spectroscopy after ^{33}S substitution

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Active xanthine oxidase was labelled specifically with ^{33}S in the cyanide-labile site of the molybdenum centre. The Very Rapid molybdenum(V) e.p.r. signal, generated from this, shows strong coupling of ^{33}S to molybdenum, providing unambiguous evidence that, at least in the signal-giving species, this sulphur atom is a ligand of molybdenum. The structure of the signal-giving species is discussed.

The molybdenum-containing enzyme xanthine oxidase may be converted on treatment with cyanide into an inactive form known as desulpho xanthine oxidase (Massey & Edmondson, 1970; Bray, 1975). Desulpho xanthine oxidase also occurs as a degradation product contaminating the active enzyme. There has been much discussion in the literature on the chemical nature of the modification to the molybdenum centre involved in this transformation. Whereas Massey & Edmondson (1970) favoured the idea that the cyanide-labile sulphur atom is present as a persulphide group, e.p.r. (Gutteridge *et al.*, 1978*b*) and X-ray-absorption-extended-fine-structure (Bordas *et al.*, 1980) studies support the proposition that the sulphur is a terminal sulphur ligand of the molybdenum atom ($\text{Mo}=\text{S}$).

E.p.r. studies after substitution with stable isotopes have provided important information on the structure and mechanism of action of the molybdenum centre of xanthine oxidase. ^2H (Gutteridge *et al.*, 1978*a,b*), ^{13}C (Tanner *et al.*, 1978) and ^{17}O (Gutteridge *et al.*, 1979; Gutteridge & Bray, 1980) have all been introduced into e.p.r.-signal-giving species from the enzyme.

Massey & Edmondson (1970) showed that the inactivation reaction caused by cyanide could be reversed, with incorporation of ^{33}S and partial restoration of enzymic activity, when desulpho xanthine oxidase was treated with radioactive sulphide. We now report re-activation of desulpho xanthine oxidase by sulphide enriched with ^{33}S , to yield active enzyme selectively labelled with this stable isotope, and describe e.p.r. studies with the product.

Materials and methods

$[^{33}\text{S}]$ Sulphide

The isotope was obtained as elemental sulphur

(from Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A.) and was converted into sulphide by a scaled-down and simplified modification of the procedure of Fehér (1963), involving reaction between sulphur and sodium in liquid ammonia. Approx. 3 ml of dry ammonia was condensed over 2 mg of sulphur at approx. -60°C . The sulphur was titrated, with precautions being taken to exclude moisture, with approx. 2.8 mg of sodium (added in the form of short lengths of freshly extruded wire) until a faint blue colour persisted in the solution. Ammonia was removed by raising the temperature gradually under a stream of dry N_2 , then evacuating for 2 h with the sample at about 20°C . The white solid was dissolved in 0.5 ml of distilled water and stored frozen under liquid N_2 in the form of beads. Both enriched (59.15 atoms % ^{33}S) and non-enriched sodium sulphide were prepared in this way.

Xanthine oxidase and desulpho xanthine oxidase

Xanthine oxidase was prepared by the salicylate denaturation procedure of Hart *et al.* (1970). Conversion into the desulpho form was described by Massey & Edmondson (1970). Though Gutteridge *et al.* (1978*b*) used extended exposure of the enzyme to cyanide to obtain the desulpho form, this was found to be unnecessary in the present studies. Indeed, in the hope of maximizing re-activation by sulphide (see below), we minimized exposure to cyanide. Conditions used were: enzyme concentration 0.3–0.7 mM [determined from ϵ_{450} (per half-molecule) = $36\text{ M}^{-1}\cdot\text{cm}^{-1}$], 10–20 mM-cyanide in 50 mM-sodium pyrophosphate buffer, pH 8.2 (measured before addition of cyanide), and an incubation time of about 30 min at 20°C . This decreased the content of functional enzyme (assayed by the method of Hart *et al.*, 1970; see also Bray, 1975) to 0.02–0.5%.

Re-activation of the desulpho enzyme with sulphide

Re-activation was achieved (Massey & Edmondson, 1970) by treating the desulpho enzyme with a solution of non-enriched or enriched sodium sulphide. Incubation with 10 mM-sulphide in pyrophosphate buffer at a final pH of 8.2–9.0 at 45°C for 1–2 h was followed by gel filtration in 50 mM-Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH 8.3. Activity measurements indicated that enzyme samples so obtained were about 30–45% functional, in agreement with earlier work (Massey & Edmondson, 1970; Edmondson *et al.*, 1972).

E.p.r. samples and e.p.r. measurements

The Very Rapid e.p.r. signal was generated by brief treatment of the re-activated enzyme with xanthine under the conditions described by Gutteridge & Bray (1980). The Slow signal was generated with dithionite (Gutteridge *et al.*, 1978b). Spectra were recorded on a Varian E9 spectrometer linked to a computer (Bray *et al.*, 1978). Field and frequency standardization was performed as described by these workers with a dual-sample cavity.

Results and discussion

Reactivation of desulpho xanthine oxidase by sulphide enriched with ^{33}S proceeded in accordance with expectations (cf. Massey & Edmondson, 1970; Edmondson *et al.*, 1972). The enzyme used was converted, initially, virtually quantitatively into the desulpho form. Thus almost 100% of the active enzyme in the re-activated samples would be labelled specifically in the cyanide-labile site with ^{33}S , at the enrichment used, namely 59 atoms %. If the cyanide-labile sulphur atom is indeed a ligand of molybdenum, then molybdenum(V) e.p.r. signals derived from the active enzyme would be modified by coupling of molybdenum to the ^{33}S nucleus. Thus, since ^{33}S has $I = \frac{3}{2}$, four-line spectra would be expected, superimposed on the ordinary spectra, with relative intensities in the ratio 59:41. On the other hand, re-activation with ^{33}S would not be expected to affect the e.p.r. of residual desulpho xanthine oxidase in the samples. In agreement with this, we found that the Slow signal generated from re-activated preparations was not distinguishable from that obtained with ordinary enzyme.

For the active enzyme, we chose to investigate effects of ^{33}S substitution by using the Very Rapid molybdenum(V) e.p.r. signal (Bray, 1975, 1980; Gutteridge & Bray, 1980). This transient signal is believed to represent an essential intermediate in turnover, and has the advantages for the present work of being uncomplicated by proton splittings and of having narrow linewidths. In Fig. 1 the

Fig. 1. E.p.r. spectra of the Very Rapid Mo(V) e.p.r. signal with ^{33}S -substituted and normal xanthine oxidase. (a) shows the Very Rapid signal produced by enzyme re-activated with non-enriched sodium sulphide, (b) shows the Very Rapid signal produced by enzyme re-activated with ^{33}S -enriched (59 atoms %) sodium sulphide, and (c) is a difference spectrum obtained by subtracting $0.8 \times (a)$ from (b), then increasing the vertical scale $\times 2$, to give a spectrum corresponding to approx. 100% enrichment by ^{33}S . Reaction conditions with xanthine (2.5 mM) were 12 ms at 21°C in 0.1 M-sodium bicarbonate buffer, pH 10.3. Traces of the Rapid signal as well as of signals from free radicals are present in (a) and (b); however, particularly the Rapid signal has largely been eliminated from (c) by the subtraction. The magnetic-field scale corresponds to 9.363 GHz. E.p.r. conditions were 120 K, 20 mW microwave power and 0.25 mT modulation amplitude.

spectrum of the Very Rapid signal from unenriched enzyme (a) is compared with that from the enriched enzyme (b). By subtracting suitable proportions of (a) from (b), spectrum (c), corresponding to 100% enrichment of the signal-giving species with ^{33}S , was obtained. Though we have not succeeded in getting good simulations (Lowe, 1978) of Fig. 1(c), probably because of non-coincidence of g - and A -tensors (Scullane *et al.*, 1979), the spectrum nevertheless shows clear signs of the fourfold features expected.

A number of points are apparent from inspection of Fig. 1(c). Firstly, ^{33}S splittings in the g_1 region are clearly quite large, about 2.6 mT (measured as indicated). However, clearly also, the A -tensor is anisotropic, A_1 being about 0.8 mT, as indicated on the Figure. The magnitude of these splittings is comparable with, or rather larger than, those found for sulphur ligands in several low-molecular-weight

transition-metal-ion complexes (Goodman & Raynor, 1970; Schmitt & Maki, 1968). Thus it seems certain that the sulphur atom detected in the Very-Rapid-signal-giving species is a ligand of molybdenum (presumably in an equatorial position) in the active enzyme.

The anisotropy of the ^{33}S splitting in the Very Rapid signal contrasts with the isotropy of the corresponding ^{17}O splitting (Gutteridge & Bray, 1980). It seems that a p -orbital contribution dominates in the bonding, with extensive delocalization of the electron on to the sulphur atom (for a p electron entirely on sulphur, an anisotropic splitting of 5.7 mT is expected; Goodman & Raynor, 1970). The anisotropy may argue for $\text{Mo}=\text{S}$ in the species responsible for the Very Rapid signal.

Combination of these conclusions with ^{17}O results (Gutteridge & Bray, 1980), as well as with X-ray-absorption-extended-fine-structure information on the oxidized enzyme (Bordas *et al.*, 1980) and with data for model compounds (Scullane *et al.*, 1979), suggests the following as the structure of the Very-Rapid-signal-giving species. Indications are that there is no terminal oxo group, the only oxygen ligand being in the form of an $\text{Mo}-\text{O}-\text{C}$ group (where C is C-8 of xanthine), bound in an equatorial position. The remaining ligands appear to be sulphur atoms, including the equatorial terminal sulphur atom revealed by ^{33}S , along with sulphur atoms (Bordas *et al.*, 1980) from two cysteine and one methionine residues. Which if any of these ligands might be supplied by the pterin 'cofactor' of xanthine oxidase (Johnson *et al.*, 1980; J. W. Williams & R. C. Bray, unpublished work) is not obvious. Clearly much further work is required to confirm this and to understand more fully the role of this species in the catalytic reaction. Work on effects of ^{33}S substitution on other e.p.r. signals (Rapid and Inhibited) from active xanthine oxidase will clearly be important.

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