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X-ray absorption spectroscopy of xanthine oxidase

The molybdenum centres of the functional and the desulpho forms

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X-ray absorption spectra have been recorded for the molybdenum *K*-edge region of xanthine oxidase. Both the absorption edge and the extended fine structure (e.x.a.f.s.) regions were investigated. Spectra were obtained for samples of the desulpho enzyme as well as for mixtures of this with the active enzyme. The spectrum of the pure active form was then obtained by difference. The desulpho enzyme shows a pronounced step in the absorption edge, of a type previously associated with terminal oxygen ligands. In the active enzyme this step has decreased markedly. Satisfactory simulations of the e.x.a.f.s. spectrum of the desulpho enzyme could be obtained by assuming the molybdenum to be bonded to two terminal oxygen atoms (Mo=O about 0.175 nm), two sulphur atoms (presumably from cysteine residues, Mo–S about 0.250 nm) and one sulphur atom (presumably from a methionine residue, Mo–S about 0.290 nm). E.x.a.f.s. of the active enzyme differed appreciably from this. In keeping with earlier proposals [Gutteridge, Tanner & Bray (1978) *Biochem. J.* **175**, 887–897], the spectrum of the active enzyme could be simulated if a sulphur atom at about 0.225 nm (i.e. presumably a terminal sulphur atom) replaced one of the terminal oxygen atoms of the desulpho form, with small changes in the other bond distances. Validity of the interpretative procedures, which involved phase shift and amplitude calculations *ab initio*, was demonstrated by using low molecular weight compounds of known structure.

Xanthine oxidase (Bray, 1975) is the most extensively studied of the molybdenum-containing enzymes (Coughlan, 1980). E.p.r. studies have been particularly revealing (Bray, 1980*b*; Gutteridge & Bray, 1980*b*) concerning the functioning of the molybdenum centre, which is the site at which reducing substrates (other than NADH) interact with the enzyme.

A peculiarity of xanthine oxidase is that, as normally isolated and studied, the enzyme is a mixture of two molybdenum-containing forms, the active enzyme and a catalytically inactive degradation product, desulpho xanthine oxidase (Bray, 1975). Though the desulpho form may be removed by affinity chromatography (Edmondson *et al.*, 1972), this separation seems never to have been achieved on anything but a very small scale.

Apart from the loss of catalytic potency, conversion of the active enzyme to the desulpho form is accompanied by marked changes in molybdenum(V) e.p.r. signals (McGartoll *et al.*, 1970; Bray, 1980*a*,*b*), by changes in the redox potential of the molybdenum (Cammack *et al.*, 1976) and by changes in absorption spectrum (Coughlan *et al.*, 1969; Edmondson *et al.*, 1972). Thus, the nature of the structural difference between functional and desulpho xanthine oxidase has been the subject of considerable work and much speculation.

It is established that a sulphur atom is liberated on conversion from the active to the desulpho enzyme (Massey & Edmondson, 1970), a natural process accelerated greatly by the presence of cyanide, which is converted to thiocyanate. Partial reactivation may be achieved with sulphide. These workers

Abbreviations used: e.x.a.f.s., X-ray absorption extended fine structure; Bicine, *NN*-bis-(2-hydroxyethyl)-glycine.

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concluded that the cyanolysable sulphur was a persulphide group in the functional enzyme. Coughlan (1977), however, suggested that the sulphur might be derived from a cysteine residue rendered labile by its proximity to molybdenum.

Gutteridge *et al.* (1978) proposed that the cyanide-labile sulphur was present as a terminal sulphur (sulphido) ligand of molybdenum in the active enzyme, replaced by a terminal oxygen (oxido) ligand in the desulpho enzyme. They also suggested that these groups would become protonated to -SH and -OH groups respectively when the enzyme forms were reduced (cf. Stiefel, 1973). This hypothesis appeared to explain both the marked difference in proton exchange rates (measured by e.p.r.) and thus in p*K* values of these groups (the active enzyme having a p*K* some 2 units lower than the desulpho enzyme) and also the parameters of the e.p.r. signals. Furthermore, the differences in redox potentials and absorption spectra were consistent with the hypothesis, which is also in accord with chemical information on model compounds (see, e.g., Williams & Wentworth, 1974; Mitchell & Pygall, 1979). Particularly, Newton *et al.* (1979) recently found that $Mo_2OS(\mu_2\text{-}S_2)(S_2CNEt_2)_2$, when treated under vigorous conditions with cyanide, formed thiocyanate and, in 71% yield, the corresponding molybdenum compound having a terminal oxido- in place of a terminal sulphido-group.

Although we are unaware of any data refuting the hypothesis of a terminal sulphido-group in active xanthine oxidase, we have remained cautious (cf. Bray, 1980*b*; Gutteridge & Bray, 1980*b*). Furthermore, Betcher-Lange *et al.* (1979) have interpreted recent inactivation data in terms of the cysteine sulphur hypothesis.

X-ray spectroscopy of metal centres in biological molecules (Chan & Gamble, 1978; Shulman *et al.*, 1978; Stern, 1978) provides a method potentially able to identify ligands of the metal and yield information about metal–ligand distances in non-crystalline samples. Two types of study are possible. The position of structure before, or in, the absorption edge can, through empirical procedures, yield information on the oxidation state of the metal, together with some indication of its immediate chemical environment. The Kronig fine structure occurring immediately beyond the absorption edge (the extended fine structure, or e.x.a.f.s. region), may be analysed by several more rigorous procedures to yield information on the nature of the co-ordinating atoms and their distances from the metal.

X-ray absorption spectroscopy has been used in the study of two molybdenum-containing enzymes, nitrogenase (Cramer *et al.*, 1978*b*) as well as the cofactor from this enzyme (Cramer *et al.*, 1978*c*) and sulphite oxidase (Cramer *et al.*, 1979). Clearly, the method should yield definitive information on the nature of the structural difference between active and desulpho xanthine oxidase and two preliminary papers have appeared (Tullius *et al.*, 1979; Bordas *et al.*, 1979). We now present a more complete study of this enzyme in the oxidized state, using both edge and e.x.a.f.s. spectra. These results, firstly, characterize the molybdenum centre of desulpho xanthine oxidase and, secondly, provide additional support for the hypothesis of Gutteridge *et al.* (1978) that conversion from the active to the desulpho form involves replacement of a terminal sulphur ligand of molybdenum by terminal oxygen.

Materials and methods

X-ray spectrometer

The X-ray spectrometer used, attached to the D.O.R.I.S., storage ring at the E.M.B.L. synchrotron radiation facility, D.E.S.Y., Hamburg, is, generally, of standard design (cf. Shulman *et al.*, 1978). Problems of harmonics and parasitic reflections have been solved by slightly offsetting the rocking curves of the crystal faces (silicon 2,2,0) in a monochromator (Hart & Rodrigues, 1978; Rodrigues, 1979) designed and developed by M. H. Hart, R. Rodrigues & D. Isaac (unpublished work). Control of the faces is achieved via a hardware servo loop monitoring continuously the degree of contamination of the beam by harmonics (P. Gill, J. Phillips & J. Bordas, unpublished work).

The X-ray flux was typically of the order of 10^{10} photons/s per eV band width (storage ring operating at 5 GeV and 20 mA), over an area of 30 mm × 1 mm, and was detected before and after sample irradiation with ion chambers filled with N_2 and Xe. The monochromator position, etc., was controlled by a C.A.M.A.C. system driven by a P.D.P. 11/45 computer.

In addition to the data in the Results section, for desulpho xanthine oxidase, simultaneous measurements of fluorescence as well as of absorption e.x.a.f.s. were made, leading to spectra essentially the same as those presented.

Enzyme samples

Milk xanthine oxidase was partially purified by the salicylate-denaturation procedure of Hart *et al.* (1970). Concentrations were determined from A_{450} and percentage functionality from activity measurements (Bray, 1975). Enzyme prepared by this method, or by other standard methods, is a mixture of the active and the desulpho forms and will be referred to as 'mixed' enzyme. Conversion of such samples completely to the desulpho form with cyanide was done by the method of Massey & Edmondson (1970). 'Mixed' or desulpho enzyme samples (about 2.5 mM in molybdenum) in Bicine/NaOH buffer (30 mM; pH 8.2) were frozen as

beads by dropping into liquid N_2 and these beads were then freeze-dried and stored at the temperature of liquid N_2, protected from moisture with silica gel, and were transported to Hamburg. For X-ray measurements, samples were pulverized and pressed into rectangular cells made of Perspex. Sample dimensions were: 3mm high and 30mm wide, with a path-length of up to about 120mm. Cell windows were transparent adhesive tape. Samples (about 2g of enzyme calculated from the A_{450}) of 'mixed' and of desulpho enzyme were studied.

Activity measurements on freeze-dried samples, exposed for various periods to temperatures of 15–23°C and irradiated for part of these times, indicated activity loss (assumed first order) corresponding to $t_{\frac{1}{2}}$ approx. 38h. After X-ray spectroscopic measurements, samples were transported, as before, at the temperature of liquid N_2, to Brighton for e.p.r. measurement of intensity of the Slow signal (McGartoll *et al.*, 1970).

Model compounds

The e.x.a.f.s. spectra of several well-characterized compounds, including [MoO_2(ethyl-L-cysteinate)$_2$] (Melby, 1969) and $(NH_4)_2MoS_4$ (Krüss, 1884), were recorded. Analytically pure samples of the compounds were finely ground and contained between transparent adhesive tape, held apart by aluminium formers, to give a uniform thickness of 1–2mm.

Measurement procedure

Samples were located carefully in the X-ray beam, exposed to the air and at a temperature of 15–23°C. For the enzyme samples, approx. 50 scans, extending from about 600eV below to 1200eV above the molybdenum *K*-edge, were recorded and averaged. Each scan took about 30min and the data from each was examined for irregularities (due, e.g., to movement of the beam) before being included for averaging. Approx. one-third of the scans contained irregularities and were omitted. For the model compounds, a single scan was generally sufficient. In all cases, a baseline with no sample present was also recorded, under the same conditions as the sample.

Data reduction and processing

This was carried out on the P.D.P. 11/45 computer used to record the data. X-ray spectra were obtained in the form of absorbance [$\log(I_0/I)$] as a function of X-ray energy in eV. Baseline absorbance was subtracted and edge spectra could then be plotted. Since the monochromator was not standardized between runs, it was necessary to judge alignment of spectra of the desulpho and 'mixed' forms of the enzyme empirically (see the Results section).

To extract e.x.a.f.s. structure from absorption to the high energy side of the edge, a fourth order polynomial, fitted to the data above the edge, was first subtracted from the data. The result was then divided by a similar polynomial, fitted to the pre-edge data. Both polynomials were constrained to extrapolate to zero absorbance at infinite energy. For the resulting normalized e.x.a.f.s. spectra, zero energy was arbitrarily taken as the inflection point in the absorption edge. Such spectra, provided that the above manipulations were carried out correctly, showed only the damped oscillations of the fine structure. When appropriate, smoothing of normalized e.x.a.f.s. spectra could be achieved by a cubic smoothing procedure. In a number of published procedures, e.x.a.f.s. spectra are plotted in terms of reciprocal distance (conversion to 'k-space') and the e.x.a.f.s. amplitudes multiplied by some power of k. However, we generally preferred to plot spectra in terms of energy and to leave them unweighted by k^n.

As indicated below, we made limited use of Fourier transforms. In obtaining these, the limited range of the data was allowed for by multiplying by a Gaussian function placed at their centre. Correction for the back-scattering factor was made before transforming (Gurman & Pendry, 1976). This procedure gives peaks in close proximity to the true positions, at the expense of an increase in line-width.

Calculation of phase shifts for computer simulation of e.x.a.f.s. spectra

This was carried out at Daresbury, by using an IBM 370/165 computer. Phase shifts were theoretically calculated with programs developed by Pendry (Pendry, 1974; Lee & Pendry, 1975; S. Gurman & J. Pendry, unpublished work). Clementi wave functions (Clementi & Roetti, 1974) and a statistical exchange term are required so that a complex potential within a 'muffin-tin' approximation (see, e.g., Ziman, 1965) can be used. For an excited molybdenum ($Z = 42$) as central atom, the wave-functions of an unscreened technetium ($Z = 43$) singly charged cation were input, along with wave-functions for neutral ligand atoms. To ensure that the phases were insensitive to the ligand environment assumed, phase shifts were calculated for molybdenum surrounded by different atoms types. Firstly, a structure similar to $[MoS_4]^{2-}$ was considered and secondly, Mo surrounded by two oxygen atoms at 0.16nm, 1 nitrogen atom at 0.23nm and 3 sulphur atoms at 0.24nm. Differences between the two sets of shifts so obtained were not significant, to the extent that, e.g., the e.x.a.f.s. of $[MoS_4]^{2-}$ could be simulated equally well by using either.

Computer simulation of e.x.a.f.s. spectra

If a number of assumptions are made, the e.x.a.f.s. region of the absorption spectrum may be expressed as an equation containing only readily determinable

quantities (Lee & Pendry, 1975; Ashley & Donaich, 1975). The corresponding computer program (Pendry, 1974; Lee & Pendry, 1975; S. Gurman, unpublished work), takes account of the sphericity of the outgoing and back-scattered waves (Lee & Beni, 1977) and of thermal disorder via a Debye–Waller term. In using this program, a number of parameters were assigned values which were not changed or were varied within small limits only, for any of the simulations presented in the Results section. These included phase shift parameters (see the previous subsection) and edge shifts (simulated spectra shifted towards higher energies by 14–22eV). Finally, to take account of inelastic scattering processes, all calculated amplitudes were multiplied by 0.8.

Further input for the simulated spectra consisted of the nature and integral number of ligand atoms assumed co-ordinated to the molybdenum and their distances from the metal, together with an assumed Debye–Waller factor for each atom. Goodness of fit of simulated to experimental spectra was judged by eye, paying particular attention to correct alignment of all the peaks in the e.x.a.f.s. spectra, as well as to the fit of the corresponding Fourier transforms. This procedure, though somewhat subjective, was preferred to statistical tests of goodness-of-fit, since these could not readily be made to take proper account of all the factors involved in this study.

For model compounds of well-characterized structure, distances and Debye–Waller factors were the only variables. They were thus useful, in the initial stages of the work, in defining the fixed parameters.

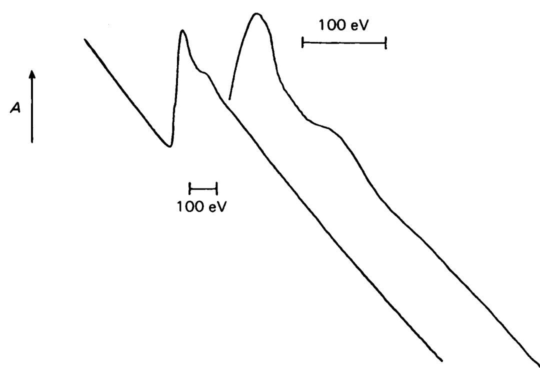

Fig. 1. *X-ray absorption spectrum of desulpho xanthine oxidase*

The curve was the average of approx. 50 scans and no smoothing has been applied to the data. The e.x.a.f.s. region is shown enlarged in the upper curve. The vertical scale corresponds to absorbance in arbitrary units and the horizontal scale to X-ray energy in eV. The molybdenum *K*-edge occurs at about 20keV.

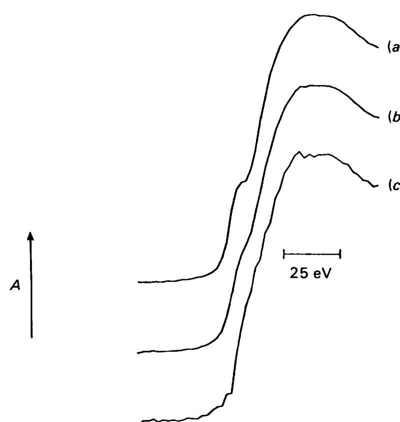

Fig. 2. *Absorption edge spectra for xanthine oxidase*

(*a*) Desulpho xanthine oxidase; (*b*) a sample containing about 40% of active enzyme; (*c*) the spectrum calculated for the pure active enzyme by subtracting 0.6 × (*a*) from (*b*) and multiplying the resultant spectrum by 2.5 (see the text concerning alignment of the spectra).

Results

Absorption edge spectra for xanthine oxidase

The unprocessed X-ray absorption spectrum of desulpho xanthine oxidase is shown in Fig. 1, with the edge region, after background subtraction, enlarged in Fig. 2(*a*). The edge region of the spectrum for a sample of mixed active plus desulpho xanthine oxidase is shown in Fig. 2(*b*) and is comparable with the spectrum reported by Tullius *et al.* (1979). For both of our samples, the edge shows a step of the type attributed, empirically, by Hodgson and co-workers to the presence of terminal oxygen ligands of molybdenum (see Tullius *et al.*, 1979; Cramer, 1977; Cramer *et al.*, 1978*b*). Clearly, however, the step in the edge of our sample of mixed enzyme is somewhat different from, and less pronounced than, that in the desulpho enzyme.

To deduce the edge spectrum of pure active xanthine oxidase from our data, it is necessary to know the relative proportions of active and inactive enzyme in the mixed sample and to align the spectra precisely. However, severe loss of activity during our experiments (see the Materials and methods section) makes precise estimation of the composition difficult. Though we started with enzyme containing 65% of active enzyme and 35% of the desulpho form, nevertheless our best estimate of the composition half-way through the scanning process, which extended over more than 1 day, is 40% active enzyme and 60% desulpho form. (See the Materials

and methods section for activity loss data. We assumed a first-order rate process and allowed for loss before, during and after irradiation.) The rate of activity loss in irradiated samples did not exceed by more than about 10% that in non-irradiated samples at the same temperature. Furthermore, e.p.r. measurements indicated a particularly strong Slow signal in mixed enzyme after the irradiation, in agreement with the assumption that activity loss in the freeze-dried state is, like that in solution at room temperature, due to conversion of active enzyme to the desulpho form. Thus, we have no reason to suspect significant contamination of active and desulpho enzyme by other molybdenum-containing species. Reduction of the enzyme by the radiation (cf. Powers *et al.*, 1979) was presumably not a problem in our work, since both forms of xanthine oxidase are likely to be readily autoxidizable at ambient temperatures, even in the freeze-dried state.

Thus, to obtain the spectrum of the pure active enzyme, it is appropriate, after normalizing the edges to the same height, to subtract 0.6 × the desulpho spectrum from that of the mixed sample and to multiply the result by 2.5. Though absolutely precise alignment may not be possible (see the Materials and methods section), the basic similarity of form of the edge spectra, and the fact that the desulpho enzyme must contribute very substantially to the spectrum of the mixed sample, reduces uncertainties greatly. Fig. 2(*c*) shows our best estimate of the edge spectrum for the active enzyme, derived from Figs. 2(*a*) and 2(*b*).

We conclude from Fig. 2 that the edge spectrum for desulpho xanthine oxidase has a prominent step whereas, in comparison, for the active enzyme the step is smaller and in a possibly slightly altered position. This suggests that active enzyme contains fewer terminal oxygen ligands of molybdenum than does the desulpho form.

E.x.a.f.s. of model compounds

Before studying the e.x.a.f.s. spectrum of xanthine oxidase we tested our manipulation and simulation procedures on a number of low-molecular-weight molybdenum compounds. We present, here, (Figs. 3 and 4) results on $(NH_4)_2MoS_4$ and $[MoO_2(ethyl\text{-}L\text{-}cysteinate)_2]$, showing the e.x.a.f.s. spectra and Fourier transforms, together with the corresponding simulations. The latter represent optimum fits obtained as described in the Materials and methods section, based on the known co-ordination geometry of the compounds but varying the distances of each ligand atom and the Debye–Waller factors. Parameters used in the simulations are summarized in Table 1. For comparison, the average bond distance for $[MoS_4]^{2-}$ obtained by X-ray crystallography is 0.218 nm (Diemann & Müller, 1973). Though crystallographic data are not available on $[MoO_2(ethyl\text{-}L\text{-}cysteinate)_2]$, bond lengths are entirely reasonable,

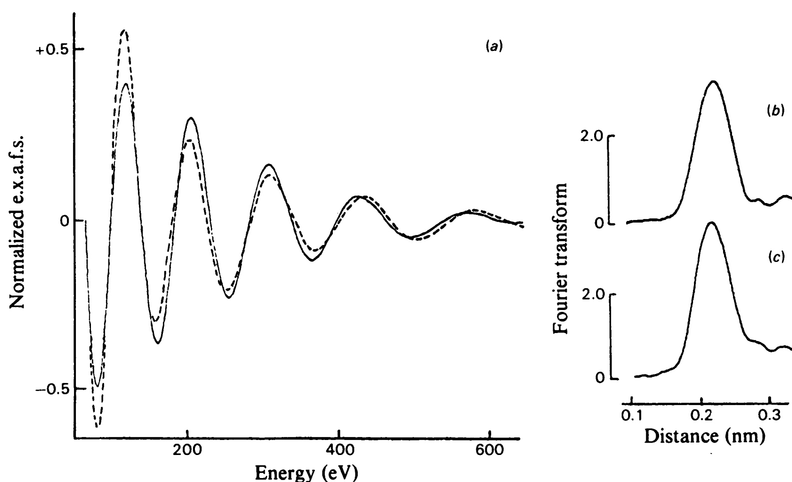

Fig. 3. *Experimental and simulated e.x.a.f.s. spectra of* $(NH_4)_2MoS_4$

Experimental and computed e.x.a.f.s. spectra and the corresponding Fourier transforms, here and in Figs. 4–6, were obtained as described in the Materials and methods section. Vertical scales correspond to unweighted normalized e.x.a.f.s. data and horizontal scales to X-ray energy in eV. Horizontal scales in the Fourier transforms represent distance in nm. Simulations employed the parameters listed in Table 1. In (*a*) the solid curve represents the simulated e.x.a.f.s. spectrum with the smoothed experimental data represented by a dashed line. (*b*) and (*c*) are the experimental and calculated Fourier transforms respectively.

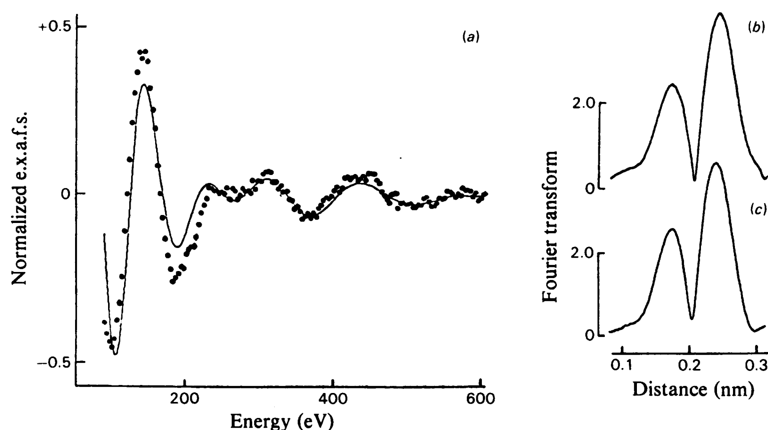

Fig. 4. *Experimental and simulated e.x.a.f.s. spectra of* [MoO_2*(ethyl-L-cysteinate)*$_2$]
Simulations employed the parameters listed in Table 1. In (*a*) the solid curve represents the simulated e.x.a.f.s. spectrum with the unsmoothed experimental data points shown as circles. (*b*) and (*c*) are the experimental and calculated Fourier transforms respectively.

Table 1. *Parameters used in simulating the e.x.a.f.s. spectra*
The values listed were obtained by the procedures described in the Materials and methods section and were used for the simulations illustrated in Figs. 3–6. Error estimates for the distances are not quoted, for reasons discussed in the text. However, ranges of distances over which acceptable fits could be obtained were approximately as follows: (*a*) Mo–S, 0.218–0.219; (*b*) Mo–O, 0.174–0.176; Mo–N, 0.237–0.240; Mo–S, 0.246–0.250; (*c*) Mo–O, 0.174–0.177; Mo–S (medium), 0.248–0.255; Mo–S (long), 0.279–0.291; (*d*) Mo–O, 0.172–0.176; Mo–S (short), 0.222–0.226; Mo–S (medium), 0.246–0.249; Mo–S (long), 0.285–0.290.

	Compound	Ligand (no.)	Distance (nm)	Debye–Waller factor
(*a*)	$(NH_4)_2MoS_4$	S (4)	0.218	0.075
(*b*)	[MoO_2 (ethyl-L-cysteinate)$_2$]	O (2)	0.174	0.018
		N (2)	0.238	0.025
		S (2)	0.248	0.030
(*c*)	Desulpho xanthine oxidase	O (2)	0.174	0.010
		S (2)	0.249	0.045
		S (1)	0.291	0.080
(*d*)	Active xanthine oxidase	O (1)	0.175	0.010
		S (1)	0.225	0.005
		S (2)	0.246	0.030
		S (1)	0.289	0.040

both on general grounds and in comparison with X-ray crystallographic data on the corresponding methyl cysteinato complex, for which the Mo–O, Mo–N and Mo–S distances are, respectively, 0.170, 0.235 and 0.241 nm (C. D. Garner, I. Buchanan & T. J. King, unpublished work).

From these and additional studies (S. S. Hasnain, J. Bordas, C. D. Garner, S. Gutteridge & R. M. Miller, unpublished work), we conclude that our procedures, when applied to known molybdenum compounds, yield bond lengths in close agreement with those derived from X-ray crystallography.

E.x.a.f.s. of desulpho xanthine oxidase

Our experimental e.x.a.f.s. data on desulpho xanthine oxidase are illustrated in Fig. 5(*a*), together with our best theoretical curve and the corresponding Fourier transforms (Figs. 5*b* and 5*c*). It is interesting to compare our parameters (as used in the simulations; see Table 1) for the ligands of molybdenum in this enzyme form with those reported by Tullius *et al.* (1979) for a sample of mixed enzyme. The agreement is surprisingly good. These workers reported oxygen atoms at 0.171 nm and sulphur atoms at 0.254 and 0.284 nm, with the

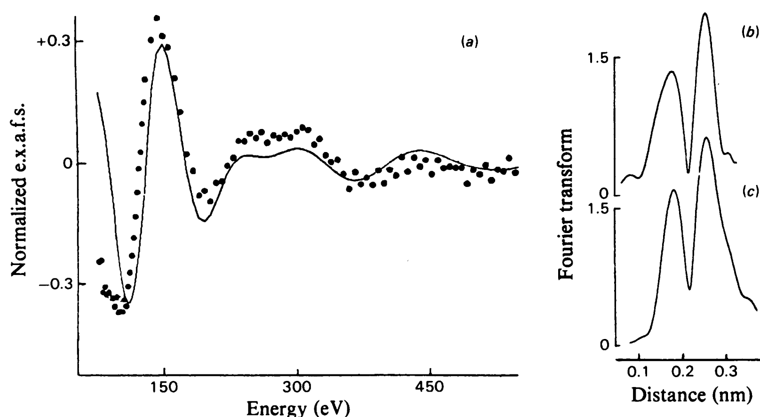

Fig. 5. *Experimental and simulated e.x.a.f.s. spectra of desulpho xanthine oxidase*
(*a*) The unsmoothed experimental points and the calculated curve based on the parameters listed in Table 1. (*b*) and (*c*) are the experimental and calculated Fourier transforms respectively.

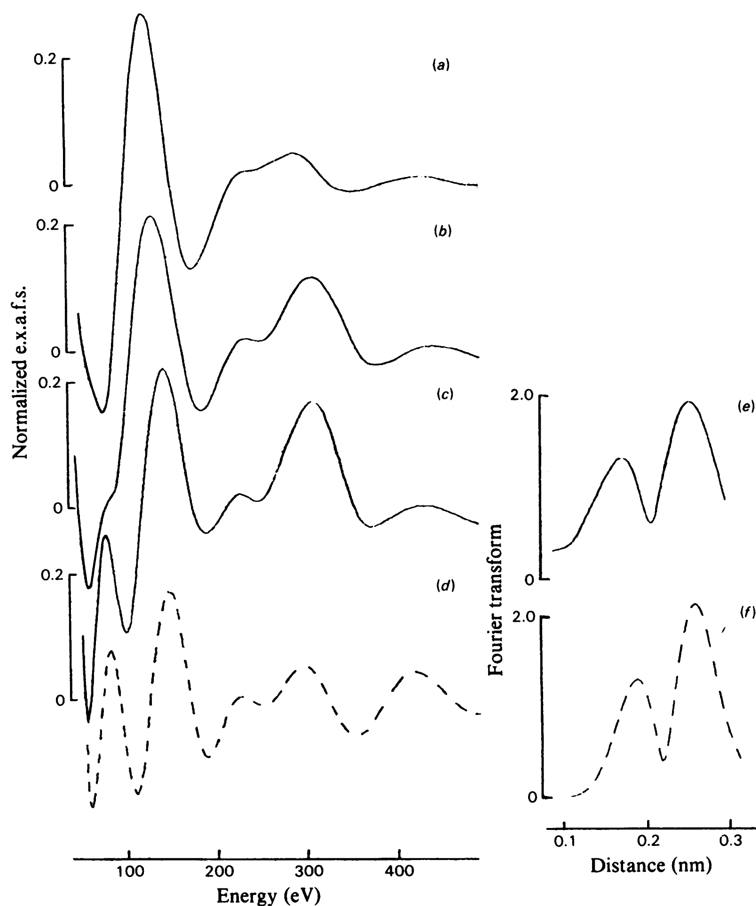

Fig. 6. *E.x.a.f.s. spectra of 'mixed' and of pure active xanthine oxidase*
(*a*) Desulpho xanthine oxidase; (*b*) a mixed sample containing about 40% active enzyme and 60% of the desulpho form; (*c*) the spectrum for pure active enzyme, obtained by subtracting 0.6 × (*a*) from (*b*) and multiplying by 2.5; (*d*) the simulation of the e.x.a.f.s. spectrum for the active enzyme. (*a*)–(*c*), Smoothed experimental curves; (*e*) and (*f*), the Fourier transforms corresponding to (*c*) and (*d*) respectively.

numbers of these ligands (to the nearest whole numbers) as 2, 2 and 1, respectively. However, it is particularly to be noted that these workers studied mixed active and desulpho xanthine oxidase, whereas our data relate to the desulpho form. This argues strongly that the spectrum of these workers was dominated by the contribution of the desulpho enzyme.

In arriving at our best simulation for desulpho xanthine oxidase we carried out computations for a variety of possible co-ordination geometries about molybdenum, including different numbers of terminal oxido-groups and allowing for the presence of nitrogen as well as sulphur ligands (cf. Fig. 4 and Bordas *et al.*, 1979). These simulations indicated the presence of two terminal oxido-groups at about 0.175nm and two sulphur atoms at about 0.250nm. However, there was little change in the form of the simulated curve if, e.g., two sulphur atoms at about 0.29nm were assumed, rather than one only. The form was also found to be somewhat insensitive to the bond length for this ligand atom.

E.x.a.f.s. spectra of mixed and of active xanthine oxidase

Figs. 6(*a*) and 6(*b*) show smoothed e.x.a.f.s. spectra for desulpho and 'mixed' (desulpho + active) xanthine oxidase, respectively. Fig. 6(*c*) shows the spectrum of the pure active form, obtained by difference from these e.x.a.f.s. spectra, by following the procedure used for subtracting the edge spectra. The three spectra show distinct differences from one another. Particularly, the mixed sample (Fig. 6*b*) shows a shoulder at about 100eV, which is not present in the desulpho enzyme (Fig. 6*a*) but which becomes a prominent peak in the active enzyme (Fig. 6*c*). This shoulder in the 'mixed' enzyme is not an artefact of the smoothing procedure, as is shown by a further comparison of the desulpho and 'mixed' enzyme samples (Fig. 7), in the form of unsmoothed e.x.a.f.s. plots from about 40 to 220eV. The shoulder is as clearly apparent in Fig. 7(*b*) as it is in Fig. 6(*b*).

Problems concerning the difference e.x.a.f.s. spectrum (Fig. 6*c*) are the same as those discussed earlier in relation to the edge spectra. Thus, precise alignment of the two spectra may not have been achieved before subtraction, and exactly the correct proportions of desulpho may not have been subtracted. Furthermore, it is possible that a minor imperfection in extraction of the e.x.a.f.s. data for one or other of the parent spectra, might have become exaggerated in Fig. 6(*c*), resulting in amplitude distortion, particularly in the low energy region. Attempts to test for the importance of these points, indicated that the absolute amplitudes, but not the positions, of the main features of the e.x.a.f.s. spectrum of active enzyme ought to be treated with caution.

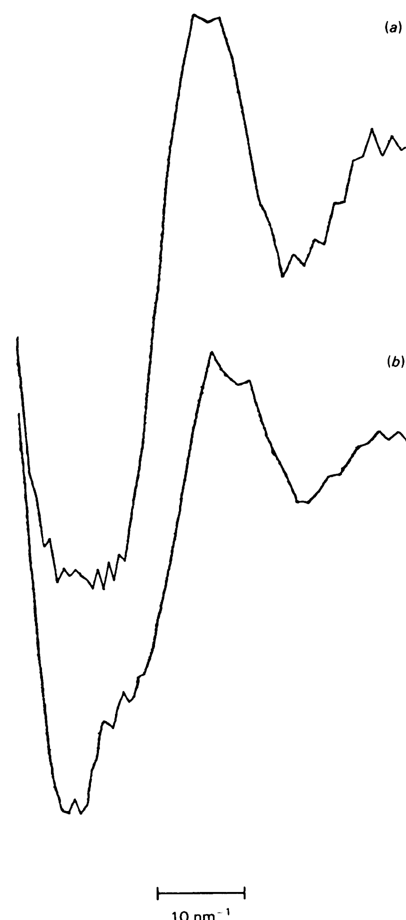

Fig. 7. *Experimental e.x.a.f.s. spectra for desulpho and 'mixed' xanthine oxidase*
The region from about 40 to 200eV is shown on an enlarged scale with unsmoothed experimental data points joined by straight lines; (*a*) desulpho xanthine oxidase; (*b*) 'mixed' xanthine oxidase (as in Fig. 6). Normalized e.x.a.f.s. data are plotted in arbitrary units with the horizontal scale corresponding to nm^{-1}.

Fig. 6(*d*) shows our best simulation of the active enzyme spectrum, based on the parameters in Table 1. In arriving at this simulation, it was found that an oxygen atom at about 0.175nm from the molybdenum, together with sulphur atoms at about 0.25nm, were sufficient to provide most of the observed features. However, particularly significantly, the only way we were able to simulate the feature at 100eV was by introducing a sulphur atom at about 0.225nm. A minor improvement of fit in the 190–380eV region was achieved if a further sulphur atom at about 0.29nm from molybdenum was included. Though Fourier transforms (Figs. 6*e* and

6*f*) supported these conclusions, we were not able to exclude altogether from the e.x.a.f.s. data, the possibility of there being two, rather than one, oxygen ligand at about 0.175 nm. Furthermore, the number of sulphur atoms at about 0.29 nm could be two rather than one.

Discussion

Interpretation of e.x.a.f.s. spectra

The procedures described in this report differ in a number of ways from those of other workers. Phase shifts and amplitudes were calculated theoretically, rather than either being dependent on Fourier transforms of the e.x.a.f.s. data, or on being obtained in an empirical manner from data on model compounds of known structure (cf. Cramer *et al.*, 1978*a*). *Ab initio* procedures are, in principle, independent of results from model compounds, whereas the alternative approach, by relying over-heavily on crystallographic information, may in some instances be inaccurate. This is because crystallographically-determined bond-lengths are susceptible to errors from 'libration' effects, whereas e.x.a.f.s., being dependent on radial distances, is insensitive to such errors.

A second point is that the calculations of earlier workers have been simplified by assuming the emitted photoelectron wave to approximate to a plane wave. This limits the e.x.a.f.s. data that can be fitted accurately to energies greater than about 100 eV above the edge. By accounting for the curved nature of the emitted and backscattered waves, our method permits data relatively close to the absorption edge to be reliably fitted.

Despite the above considerations, our results are subject to the normal limitations of e.x.a.f.s., namely that the numbers of atoms at particular distances cannot be specified with certainty. This is especially so for those ligand atoms that are more remote from the central atom. In conformity with chemical reality, we restricted our simulations to integral numbers of ligand atoms, though no doubt, had we allowed fractional occupancy (cf. Cramer *et al.*, 1978*a*; Tullius *et al.*, 1979), this would have yielded better fits of the simulations.

Structure and mechanism of action of xanthine oxidase

Our 'mixed' active and desulpho enzyme samples contained rather low and somewhat uncertain quantities of active enzyme. This and alignment problems make difference data relating to active enzyme less certain than are data relating to the desulpho form. Nevertheless, we do not think that these problems affect the main conclusions of the work.

Molybdenum co-ordination in the desulpho enzyme appears to consist of two terminal oxido-groups, two sulphur atoms presumably from cysteine residues, at a bond distance of about 0.250 nm, and another sulphur atom at a longer bond distance and presumably from a methionine residue.

Our edge data show clearly that the two enzyme forms have a difference in their edge steps, the nature of which is consistent with a decrease in terminal oxygen ligands in active enzyme in comparison with the desulpho form. Though terminal sulphur, as well as terminal oxygen, atoms produce steps in the edge spectra of molybdenum compounds (Cramer, 1977; Cramer *et al.*, 1978*c*), it seems that those due to sulphur are weaker than those due to oxygen. There may also be some support from the e.x.a.f.s. data for one, rather than two, terminal oxygen ligands in the active enzyme. Furthermore, the e.x.a.f.s. of the active enzyme provides clear evidence for a sulphur atom about 0.225 nm from molybdenum. Such a distance is relatively long for a terminal sulphur ligand of molybdenum, typical values for such a bond ranging from 0.21 nm (Huneke & Enemark, 1978) to 0.22 nm (Diemann & Müller, 1973). Nevertheless, the observed bond length is far shorter than could reasonably be expected for a molybdenum(VI)–cysteinyl or –persulphide sulphur, for both of which the shortest approach is about 0.24 nm (Stiefel, 1977; Müller *et al.*, 1979). Apart from the above differences, the indications from e.x.a.f.s. data are that other ligands are essentially the same in the two enzyme forms.

Thus, the work, though not completely definitive, supports the proposal of Gutteridge *et al.* (1978) that the main difference between active and desulpho xanthine oxidase is replacement of terminal sulphur in the active enzyme by terminal oxygen, the sulphur atom involved being cyanide-labile. Further work on the role of this sulphur atom in the turnover processes of the enzyme is required (Gutteridge & Bray, 1980*a*; J. P. G. Malthouse & R. C. Bray, unpublished work).

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