Information from e.x.a.f.s. spectroscopy on the structures of different forms of molybdenum in xanthine oxidase and the catalytic mechanism of the enzyme

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X-ray spectroscopy was used to provide further information on the structure of the molybdenum centre of xanthine oxidase. Earlier work was confirmed and two states of the enzyme, not reported on by previous workers, were studied. One of these was the complex of the enzyme with pyridine-3-carboxaldehyde, in which most of the metal is in the Mo(V) state, giving the e.p.r. signal known as Inhibited. The other was the complex with the inhibitor alloxanthine, with the metal as Mo(IV). For both complexes clear evidence was obtained that an oxo ligand of molybdenum was present, but not a sulphido ligand. This information complements structural information on these complexes already available from e.p.r. spectroscopy, and has permitted us to revise and refine the structures previously proposed. The mechanism of action of the enzyme is discussed in the light of the present findings on the persistence of the oxo group in the reduced enzyme complexes, as well as of related evidence [George & Bray (1988) *Biochemistry* 27, 3603–3609] for an oxo group in the catalytic intermediate that gives the Mo(V) e.p.r. signal known as Very Rapid.

INTRODUCTION

Xanthine oxidase is the most extensively studied of those molybdoenzymes (Coughlan, 1980; Spiro, 1985) that are dependent on the molybdenum cofactor. Structural information (Bray, 1988) on the molybdenum centre and its action in catalysis by the enzyme has been obtained by two complementary spectroscopic methods, e.p.r. and e.x.a.f.s. Such work has been facilitated by comparison with low- M_r compounds that have become available in the last few years, and serve as models for aspects of the enzyme's properties. Molybdenum in the enzyme can exist in Mo(VI), Mo(V) and Mo(IV) oxidation states. Only Mo(V) is observable by e.p.r., and conversion into this state is rarely quantitative. A feature of the e.p.r. studies is the finding that the enzyme can exist in a large number of distinct and clearly defined states of ligation of its molybdenum in the quinevalent state. Individual Mo(V) species correspond to substrate complexes, product complexes, an intermediate in catalysis, and complexes with inhibitors and other molecules or ions. Substantial structural information has been forthcoming (e.g. Bray & Gutteridge, 1982; Bray & George, 1985; George & Bray, 1988) from analysis of the e.p.r. parameters of these species, and in particular from analysis of ligand hyperfine couplings. In contrast with analysis by e.p.r., analysis of molybdenum ligation in the enzyme by e.x.a.f.s. provides only averaged information if mixtures of species are present, but is applicable to all oxidation states of the metal. Thus it is particularly valuable in providing information about Mo(VI) and Mo(IV), which predominate over Mo(V) in most enzyme samples. E.x.a.f.s. has been particularly important (cf. Bray, 1984) in identifying oxo (Mo=O) and sulphido (Mo=S) ligands in the enzyme.

There have been a number of studies by e.x.a.f.s. of milk xanthine oxidase and the closely related avian xanthine dehydrogenase, more recent ones being those of

Bordas *et al.* (1980), Cramer *et al.*, (1981) and Cramer & Hille (1985). E.p.r. information [Barber *et al.* (1976); obtained on Mo(V) states of the enzymes, but presumably extrapolatable to Mo(IV) and Mo(VI) states also] suggests that the molybdenum centres of the oxidase and the dehydrogenase are likely to be indistinguishable by e.x.a.f.s. Nevertheless, it was important to carry out the further studies described below. This was partly because the composition and oxidation state of some of the enzyme samples employed in earlier work did not seem to have been adequately defined. It was also because, to understand the enzyme further, we wished to study xanthine oxidase species not previously investigated by e.x.a.f.s. Throughout the work, we took stringent precautions to maximize conversion of the enzyme into single species of molybdenum, employing e.p.r. monitoring of our samples at all stages. A preliminary report on the work has been presented (Turner *et al.*, 1988).

MATERIALS AND METHODS

Xanthine oxidase samples

Xanthine oxidase was purified from milk by procedures based on those of Hart *et al.* (1970) and Nishino *et al.* (1981). Enzyme samples, for conversion into the desulpho form, were prepared by using denaturation with sodium salicylate (procedure H1,2 of Ventom *et al.*, 1988), then treated with cyanide as described by Massey & Edmondson (1970). Functional enzyme, low in the desulpho form, was prepared by affinity chromatography (procedure H1;N1,2 of Ventom *et al.*, 1988). The content of residual desulpho enzyme was monitored by measuring intensity of the Slow Mo(V) e.p.r. signal, as described by these workers. All samples were prepared in 50 mM-Bicine/NaOH buffer, pH 8.2, containing 1 mM-EDTA and concentrated by ultrafiltration (Minicon B concentrators from Amicon). Samples of up to 2.0 mM-

xanthine oxidase final concentration (from A_{450} with ϵ 36000 M⁻¹·cm⁻¹) were used. After appropriate treatments, they were filled into e.x.a.f.s. cells (approx. 4 mm × 6 mm × 14 mm, with thin Mylar windows), frozen, and transported to Daresbury at about 77 K. Where indicated, reduced samples were prepared and frozen in an anaerobic glove cabinet, operating at 1 p.p.m. of O₂.

Individual enzyme samples were prepared as follows. Oxidized functional and desulpho samples used the enzyme as prepared. The functional form contained 10% of residual desulpho enzyme. This value was obtained before concentration. On the e.x.a.f.s. sample, thawed after running, the value had risen to 14%. Reduced desulpho enzyme was obtained by treating it, in the glove cabinet, with 30 mM-dithionite, the time course of the reduction being monitored by e.p.r. (cf. Bray *et al.*, 1975). The Slow Mo(V) e.p.r. signal reached maximum intensity after 10 min at 20–25 °C, then decreased slowly. The sample was frozen after 16 h, when the signal corresponded to 0.5% of the total molybdenum.

The alloxanthine complex of the functional enzyme was obtained by reduction with 20 mM-dithionite for 20 min at 20–25 °C, followed by incubation with alloxanthine (10 mM; a gift from the Wellcome Foundation, Dartford, Kent, U.K.). The progress of the inhibitory reaction was followed by measuring xanthine oxidase activity of appropriately diluted samples. Initial rates were measured, since activity increased with time following dilution into the assay mixture (Williams & Bray, 1981). The sample was frozen after 30 min, when less than 1% of the activity remained. The only e.p.r. signal detected in this sample was a very weak Slow Mo(V) signal.

The Inhibited Mo(V) species was generated by treatment (Morpeth & Bray, 1984) of the functional enzyme with pyridine-3-carboxaldehyde (150 mM) at 4 °C. The sample was stirred gently in an open vessel and O₂ was bubbled through it occasionally. E.p.r. measurements, on a sample withdrawn from the mixture after 45 min reaction time, indicated that only the Inhibited Mo(V) signal was present, at an intensity corresponding to 68% of the total molybdenum. After 50 min, the bulk of the sample was transferred to an e.x.a.f.s. cell and frozen. E.p.r. on the sample in the cell after the e.x.a.f.s. had been recorded, gave a signal intensity corresponding to 63% of the molybdenum.

E.p.r. measurements

A computer-interfaced Varian E9 instrument was employed. Integrations, for calculation of percentage conversion of molybdenum into Mo(V), were performed with Cu²⁺-EDTA as standard, with corrections as in earlier work (e.g. Barber *et al.*, 1976), and assumed 1 mol of Mo/mol of enzyme (Ventom *et al.*, 1988). In some cases samples were monitored directly in the e.x.a.f.s. cells, by using a Varian V4535 wide-access cylindrical sample cavity with 25 mm-diam. sample-access holes. E.p.r. running conditions were generally 120 K, 10 mW microwave power and 0.16 mT modulation amplitude.

X-ray spectra

Mo K-edge X-ray fluorescence spectra were recorded on the wiggler beamline, Station 9.2, of the Synchrotron Radiation Source, Daresbury Laboratory, operating at 2 GeV and 100–200 mA, with a silicon [2,2,0] double-

crystal monochromator. Four NaI detectors with Zr filters of appropriate thickness (cf. Baines *et al.*, 1986) were employed. An Mo foil standard, placed in series after the sample, and with its X-ray absorption recorded simultaneously with the data by an additional ion chamber, was employed for calibration and for precise alignment of individual spectra. The position of the first post-edge minimum on the first derivative of the Mo-foil absorption spectrum was taken as 20022.5 eV. Samples were maintained at 60–90 K during measurement. A monochromator entrance vertical slit height of 0.7 mm was used to provide adequate resolution for edge spectra, increased to 1.6 mm for the e.x.a.f.s. region. For the latter, approx. 1 h scans were used; six scans were averaged for the most concentrated samples (2.0 mM-Mo) and up to 16 for the most dilute (1.1 mM-Mo). In averaging spectra, individual detector outputs were checked and weighted in proportion to edge heights; any showing irregularities were omitted from the average. Small shifts, as determined from the Mo foil, were made to the spectra where necessary to compensate for monochromator drift.

E.x.a.f.s. data manipulation and interpretation

Manipulation of the data, extraction of the e.x.a.f.s. and simulation were performed with standard Daresbury Laboratory software. Background subtraction and normalization used conventional procedures (see, e.g., Scott, 1984); the program EXBROOK was used. No smoothing or filtering was applied to the averaged data. E.x.a.f.s. spectra are plotted in terms of the parameters k , in Å⁻¹, with the origin as defined in Table 1; amplitudes are, however, scaled as for atomic units. Simulation, based on curved-wave single-scattering theory, employed the program EXCURV86 (see Gurman *et al.*, 1984; Joyner *et al.*, 1987). In all cases the simulations quoted below correspond to optimized fits obtained after trying a wide range of possible co-ordination spheres. We used phase and amplitude functions kindly made available by Professor C. D. Garner and Dr. A. Flood (Flood, 1986). These had been theoretically calculated by using the program MUFPO, with empirical adjustment of parameters, particularly the muffin tin radius, so as to obtain good fits to model compounds. These workers tested, in their work (Flood, 1986), a considerable number of Mo complexes for which crystallographic information was available, finding for example in 12 mononuclear Mo compounds with N, O, S or halide ligands that Mo=O bond lengths determined by e.x.a.f.s. were on average 0.1% shorter than the values determined crystallographically, the standard deviation for the difference between the two sets of values being 1.2% and the maximum deviation 2.6%. In the present work, for this bond in Na₂MoO₄ we found 1.741 Å (i.e. 0.1741 nm), and in [Et₄N][MoO(SPh)₄] 1.694 Å (i.e. 0.1694 nm), these values being respectively 1.1% shorter and 1.5% longer than the crystallographic values. The EXCURV86 parameters VPI and AFAC were fixed at -4.0 eV and 0.80 respectively.

RESULTS

Comparison with earlier data

Xanthine oxidase in five different states was examined by X-ray spectroscopy; our results are summarized in Figs. 1–3 and Table 1. Typical edge spectra are shown in

Fig. 1. Molybdenum *K*-edge X-ray fluorescence edge spectra of different forms of xanthine oxidase

Samples were prepared and spectra recorded as described in the Materials and methods section. (a) is from the oxidized functional enzyme, (b) from a sample giving the Inhibited signal, (c) from the alloxanthine complex and (d) from the oxidized desulpho enzyme.

Fig. 2. Molybdenum *K*-edge X-ray fluorescence spectra of different forms of xanthine oxidase, showing unprocessed e.x.a.f.s. data

Samples were prepared and spectra recorded and averaged as described in the Materials and methods section. No smoothing or filtering has been applied to the data; spectra were routinely recorded between 19900 and 21000 eV, but only the range 20000–20300 eV is illustrated. (a) is from the oxidized functional enzyme, (b) from a sample giving the Inhibited signal and (c) from the alloxanthine complex.

Fig. 1, typical raw data for e.x.a.f.s. in Fig. 2, and k^3 -weighted e.x.a.f.s. and Fourier transforms, together with the best simulations, in Fig. 3. Data manipulation and simulation were performed as described in the Materials and methods section. Table 1 summarizes the parameters used for the fits that are illustrated, as well as for other fits, and lists data from the literature for comparison.

Three of our samples, i.e. oxidized and reduced desulpho xanthine oxidase and oxidized functional xanthine oxidase, have all been reported on previously in

the literature. In general, though there are no substantial discrepancies, our results should be more reliable than the earlier ones, either because the e.x.a.f.s. has been analysed to rather further from the absorption edge (see Table 1), or because the state of the samples has been better characterized, or both. Serious shortcomings in earlier studies were a limited data range (4.0–11.5 Å) in the work of Bordas *et al.* (1980) and substantial contamination of the functional enzyme by the desulpho form in the experiments of these workers and in those of Cramer *et al.* (1981). The latter problem was minimized in the current work, as in that of Cramer & Hille (1985), by using enzyme purified by affinity chromatography. However, our use of e.p.r. to estimate residual desulpho enzyme is in principle more specific than the enzyme activity assays used for this purpose by Cramer & Hille (1985). In the case of the reduced desulpho enzyme, a potentially more serious problem relates to the work of Cramer *et al.* (1981). These workers reduced the avian dehydrogenase with dithionite for an unspecified reaction time; Bray *et al.* (1975) reported that (for the milk enzyme) this reduction process is very slow, taking up to 12 h or more for completion. Since Cramer *et al.* (1981) did not use e.p.r. to monitor reduction, there can be no guarantee that their sample was fully reduced. In contrast, in the present work we checked on this point, as described in the Materials and methods section.

Notwithstanding these points and that different storage rings and somewhat different software were used, the agreement with the earlier work is in general good (Table 1). Major points of agreement include the finding of one oxo and one sulphido ligand in the functional enzyme, changing to two oxo ligands in the desulpho form. On reduction of the latter, one oxo group is lost and the other remains. The agreement between the present and earlier work regarding the –SR ligands (which provide the largest feature in the Fourier transforms) is particularly good, regarding both bond lengths and Debye–Waller factors. There is a notable shortening of the average Mo–SR bond length on reduction of the desulpho enzyme, this presumably being due to loss of an oxo group *trans* to an –SR (the so-called ‘*trans* effect’: cf. Yamanouchi & Enemark, 1979). The nature and bond distances for the third (or fourth) ligand atoms (indicated in the column of Table 1 headed Mo–OR/NR), which are included to improve the fits, are the least reliable of the parameters determined in this and in earlier work. The significance of variations in them (Table 1) is thus not certain, as is the indication for the reduced desulpho enzyme [see the Fourier transform in Fig. 3(b) and footnote ‡ to Table 1] of an additional relatively distant ligand. On the other hand, we find Mo=O bond lengths that are, for both oxidized enzyme forms, some 0.05 Å longer than in the earlier work, a discrepancy that seems beyond the limits (cf. Flood, 1986; see also the Materials and methods section) of expected errors.

In view of the importance of the sulphido group in the functional enzyme (Bray, 1988), and since simulations confirmed that it is not likely to show a peak in the Fourier transform that is resolved from that of –SR groups, we sought further confirmation of its presence. We first did a plot (not shown) of assumed Mo=S bond length versus fit error parameter. In agreement with Cramer *et al.* (1981), a very sharp minimum was found at the length (2.18 Å) that is indicated in Table 1. We further checked, similarly, on the number of sulphido

ligands, finding (Fig. 4) a fairly sharp minimum in the error parameter for 1.0 such ligand atoms.

Studies on the Inhibited species and on the alloxanthine complex of xanthine oxidase

We examined two further forms of the enzyme, for which e.x.a.f.s. studies have not previously been reported. Aldehyde substrates of the enzyme undergo (Pick *et al.*, 1971; Malthouse *et al.*, 1981a; Morpeth & Bray, 1984)

during turnover a side reaction with the enzyme that leads to reversible loss of enzymic activity, accompanied by appearance of a Mo(V) e.p.r. signal, given the name Inhibited. If pyridine-3-carboxaldehyde is used as substrate, very high conversion of the enzyme into the signal-giving species is obtained (Morpeth & Bray, 1984), and we have now examined, by e.x.a.f.s., a sample in which approx. 65 % of the total molybdenum was present as the Inhibited Mo(V) signal-giving species. We also studied the complex of the functional enzyme with the potent inhibitor alloxanthine. In this complex (Massey *et al.*, 1970) the metal is primarily in the Mo(IV) state, though after treatment with oxidizing agents (Hawkes *et al.*, 1984) a small proportion of the molecules have the metal in the Mo(V) state.

The most striking feature of our results (Figs. 1b, 1c, 2b, 2c, 3d, 3e and 4 and Table 1) on these two samples is the clear evidence they provide, in each case, for the presence of an oxo ligand and the absence of a sulphido ligand. Thus the edge spectra retain the 'step', which is well known (cf. Cramer *et al.*, 1978) to be characteristic of oxo groups, at about the same intensity as in the oxidized functional enzyme (Fig. 1a); in the oxidized desulpho enzyme, with two oxo groups, the 'step' is much more pronounced (Fig. 1d; cf. Bordas *et al.*, 1980; Cramer *et al.*, 1981). The e.x.a.f.s. Fourier transforms and optimized fits (Fig. 3 and Table 1) support the presence of an oxo ligand in these samples.

In the sample giving the Inhibited e.p.r. signal, there was only 65 % conversion of the total molybdenum into the signal-giving species. Of the remaining 35 %, 10–14 % is known to be desulpho enzyme (presumably in the oxidized state and having two oxo groups), with the remainder in a state that most likely corresponds (Morpeth & Bray, 1984) to a Mo(IV) analogue of the Mo(V) Inhibited species. We therefore thought it important to quantify the oxo content of the enzyme in the sample as precisely as possible. As shown in Fig. 4, the e.x.a.f.s. fit was optimum for 1.0 oxo groups; attempts (not illustrated) to optimize the fit for 0.2 oxo groups (to correspond to the content of desulpho enzyme) gave substantially worse fits.

For the alloxanthine complex [again having 10–14 % of the molybdenum as the desulpho form, but with the remaining 86–90 % presumably as a single Mo(IV) species], Fig. 4 shows plots of the error parameter as a function of oxo ligand numbers, for two different values of σ , these being respectively the lowest and the highest

Fig. 3. E.x.a.f.s. spectra (k^2 -weighted) and Fourier transforms for different forms of xanthine oxidase

Samples were prepared and spectra recorded, processed and simulated as described in the Materials and methods section. Continuous lines correspond to experimental data, and broken lines to best simulations obtained with the parameters given in Table 1. (a) corresponds to the oxidized desulpho enzyme, (b) to reduced desulpho enzyme, (c) to oxidized functional enzyme, (d) to a sample giving the Inhibited signal and (e) to the alloxanthine complex. The e.x.a.f.s. spectra are displayed in k space (units of $\text{\AA}^{-1}$; see the Materials and methods section). To the right of the e.x.a.f.s. spectra are the corresponding phase-corrected Fourier transforms, showing the transform magnitude (vertical scale) plotted as a function of radial distance, r , in $\text{\AA}$.

Table 1. Data from e.x.a.f.s. curve-fitting: ligand of Mo in xanthine oxidase in different states

Curve-fitting, as illustrated in Fig. 3, was carried out as described in the Materials and methods section. The numbers (constrained to integral values) and distances (r) of the different ligand atoms used in the fits are indicated, together with the values of σ , from $2\sigma^2$, the Debye-Waller-like term in the e.x.a.f.s. equation. The origin of the experimental curves was taken (in defining k) as $20015.7 \text{ eV} \pm$ the shifts indicated, and that of the simulated curves was $19998.7 \text{ eV} \pm$ the shifts indicated. The fits were optimized over data ranges of from $4.0 \text{ \AA}^{-1}$ to the values indicated; all fit error parameters are quoted for k^2 -weighting and a range of $4\text{--}14 \text{ \AA}^{-1}$.

Sample	Data range ($\text{\AA}^{-1}$)	Origin shift (eV)		Mo=S		Mo=O		Mo-SR		Mo-OR/NR		Fit error parameter	Reference
		Experimental	Simulation	No.	r ($\text{\AA}$)	σ	No.	r ($\text{\AA}$)	σ	No.	r ($\text{\AA}$)	σ	
Desulpho (ox.)	14.0	+3.9	+3.9				2	1.73	0.055	2	2.48	0.084	Present work Cramer <i>et al.</i> (1981) [†]
	13.0						2	1.67	0.039	2	2.46	0.072	
Desulpho (red.)	14.5	-3.3	-0.8				1	1.68	0.071	2	2.35	0.063	Present work Cramer <i>et al.</i> (1981) [†]
	13.0						1	1.66	0.060	3	2.33	0.062	
Functional (ox.)	14.0	-0.2	+0.4	1	2.17	0.063	1	1.73	0.071	2	2.46	0.071	Present work Cramer & Hille (1985) Cramer <i>et al.</i> (1981) [†]
	14.0	-0.2	+0.4	1	2.18	0.071	1	1.74	0.063	2	2.47	0.071	
	13.0			1	2.15	0.026	1	1.67	0.039	2	2.44	0.073	
	14.0			1	2.15	0.054	1	1.70	0.028	2	2.46	0.070	
Inhibited species§	13.5	-1.6	-1.1				1	1.71	0.055	2	2.39	0.063	Present work
Alloxanthine complex	16.0	-0.5	-2.5				1	1.70	0.063	3	2.36	0.055	
										1	2.34	0.063	Present work
												1.07	Present work

* Error parameters for the desulpho enzyme are larger than, and not strictly comparable with, those from other samples, since different numbers of points were used for the two series of spectra.

† Data for xanthine dehydrogenase, not xanthine oxidase.

‡ The rather poor fit is related to the additional peak in the Fourier transform (Fig. 3b) and was improved if additional shells were included, e.g. to 2.03 for an Mo-SR at $2.81 \text{ \AA}$.

§ 65% conversion into Mo(V) species.

|| Constrained to this value (cf. oxidized functional enzyme).

Fig. 4. Numbers of oxo or sulphido ligands in different xanthine oxidase species

The fit error parameter, as calculated by the program EXCURV86 (see the Materials and methods section), is plotted for the e.x.a.f.s. data of Figs. 3(c)–3(e) as a function of the assumed number of ligand atoms of the type indicated. — corresponds to variation in the number of =S ligands for the oxidized functional enzyme, --- to variation of the number of =O ligands for the sample giving the Inhibited signal, ---- to progressive replacement of the =O group of the Inhibited species by a =S group (σ 0.071), — to variations of the number of =O ligands for the alloxanthine complex (σ 0.071) and ----- to the same variation, but with σ 0.055. The starting point, corresponding to the ● symbols on the graph, was generally the best fit of Table 1; only the ligation number (or numbers) indicated were varied, all other parameters remaining as in Table 1. For the alloxanthine complex the starting point was again the best value from Table 1, except that two values of σ were used for the oxo group, these being respectively the highest and the lowest found for such a group in the present work (Table 1). The fit ranges used varied from sample to sample (Table 1).

found in the present work for an oxo group in xanthine oxidase samples (Table 1). With the former value the fit was optimal with 0.6–0.7 of an oxo group, and with the latter with 0.8–0.9. A still further increase in σ to 0.077 (not illustrated) was sufficient to bring the minimum close to 1.0 oxo groups. Thus, from the curve-fitting, although the oxo contribution clearly seems too big to be accounted for by contamination with the desulpho enzyme, on the other hand we cannot completely exclude the possibility that a significant proportion of the functional enzyme molecules present as the alloxanthine complex in our sample contain no oxo group. However, both the magnitude of the ‘step’ in the edge (Fig. 1c) and

the amplitude of the oxo peak in the Fourier transform (Fig. 3e), which is very similar to that for the Inhibited sample (Fig. 3d), must also be considered. Bearing these in mind, we conclude that the Mo(IV) alloxanthine complex, or at least the dominant species of this if it should be that more than one form exists, without doubt bears an oxo ligand group.

Finally, Fig. 4 illustrates our attempts, which were quite unsuccessful, to find indications of the presence of sulphido ligands in these samples. As is shown for the sample giving the Inhibited signal, progressive replacement, in the simulation, of the oxo group by a sulphido group gave a rapidly worsening fit to the e.x.a.f.s. data.

DISCUSSION

Structures of reduced xanthine oxidase species bearing bound substrate or inhibitor residues

Scheme 1 summarizes structural information from our new e.x.a.f.s. data, as well as from earlier e.p.r. work, on three reduced xanthine oxidase species, each of which is known to bear a residue from a substrate or an inhibitor molecule bound, presumably covalently, to the molybdenum centre.

For the Inhibited species, estimates have been made of hyperfine couplings of Mo(V) to ^{33}S (introduced via the cyanide-labile site; Malthouse *et al.*, 1981b) and to ^{17}O (introduced by exchange with labelled water; Bray & Gutteridge, 1982), as well as to the ^1H and ^{13}C (Tanner *et al.*, 1978) of the inactivating molecule when this is formaldehyde or methanol. The structure we now propose for this species [already foreshadowed (see George, 1983; Bray, 1988)] is illustrated in Scheme 1(a). In Scheme 1 the values of the hyperfine couplings in mT are written beside the atoms to which we assign them, and atoms detected by e.x.a.f.s. are shown ringed (failure to detect every ligand by e.x.a.f.s. is not unexpected; cf. Flood, 1986).

The cyclic structure of Scheme 1(a) is compatible with the stability of the Inhibited species. In possible alternative cyclic structures (Bray & Gutteridge, 1982; Morpeth & Bray, 1984), the sulphido ligand of the oxidized enzyme remains intact and the aldehydic carbon is linked to molybdenum via two oxygen atoms. However, our e.x.a.f.s. results now show that it is the oxo not the sulphido group of the oxidized enzyme that survives in the Inhibited species. The work of Bray & Gutteridge (1982) showed there were two oxygen atoms weakly coupled to molybdenum and not exchangeable with the solvent. This is compatible with the structure illustrated, provided that it can be accepted that the oxo group does not exchange readily with the solvent. There is now a considerable body of data (Hinch *et al.*, 1986) on oxygen exchange in low- M_r molybdenum compounds with which to compare data on the enzyme; half-times for exchange of oxygen atoms in different types of sites range from a few seconds to several years. According to this work, for an oxo group to exchange rapidly with the solvent it must have *cis* to it a ligand that facilitates the exchange process. A *cis* water molecule fulfils this role, alternatives being an OH^- ion, or indeed any ligand that readily exchanges with water, or another oxo group. In the Inhibited species, evidence against such a site for water co-ordination is provided by the complete insensitivity (Morpeth & Bray, 1984) of the e.p.r. parameters of the signal obtained with a particular aldehyde to variations

Scheme 1. Proposed structures, from e.x.a.f.s. and e.p.r. data, of reduced xanthine oxidase species bearing bound substrate or inhibitor residues

Ligand atoms detected in the current e.x.a.f.s. work are ringed; numbers are hyperfine couplings in mT, estimated from e.p.r. work (see the text for references). For the Inhibited species, both e.p.r. and e.x.a.f.s. data relate to the Mo(V) state. (The species shown is for formaldehyde as the inactivating aldehyde; with other aldehydes H would be replaced by the appropriate R group). For alloxanthine, e.x.a.f.s. data relate to the Mo(IV) complex and e.p.r. data to the Mo(V) complex. No e.x.a.f.s. has been reported on the Very Rapid Mo(V) species, and the two structures shown are the alternatives proposed from e.p.r. by George & Bray (1988).

in the buffer. Such insensitivity is rather unusual among molybdoenzyme Mo(V) species (cf., e.g., Gutteridge *et al.*, 1978*a,b*; George *et al.*, 1989) and seems fully to explain the lack of oxygen exchange of the oxo group of the Inhibited species, consistent with the structure of Scheme 1(a).

Another feature of the Inhibited species is the relatively strong hyperfine coupling to carbon (Tanner *et al.*, 1978). In support of the four-membered ring structure of Scheme 1(a), this coupling seems analogous to strong ^{31}P coupling in Mo(V) dithiophosphinato complexes (Stiefel *et al.*, 1977). Note also that in the proposed structure the aldehydic hydrogen has been oxidized to a hydroxy group. Evidence from ENDOR (electron-neutron double-resonance) work (Pinhal *et al.*, 1989) favouring this conclusion, which is not in accordance with the proposals of Morpeth & Bray (1984), is being published separately.

The alloxanthine complex of xanthine oxidase has been studied in the Mo(V) state by e.p.r. (Hawkes *et al.*, 1984) and now in the present work in the Mo(IV) state by e.x.a.f.s. The structure for the Mo(IV) species of Scheme 1(b) is in accordance with our e.x.a.f.s. results, which are, in turn, in agreement with independent e.x.a.f.s. measurements on the same species by Hille *et al.* (1989). For the Mo(V) species Hawkes *et al.*

(1984) concluded, from the ^{33}S and ^{14}N hyperfine couplings, that a sulphido ligand was present and that the N-2 atom of the alloxanthine molecule was ligated to molybdenum, as shown in Scheme 1(c). However, these workers did not predict the presence of the oxo group, whose inclusion in the Mo(V) structure as shown is necessitated by extrapolation from the e.x.a.f.s. results on the Mo(IV) species. Indeed, as is discussed further below, Hawkes *et al.* (1984) failed in their e.p.r. studies to find any evidence for coupling of ^{17}O to molybdenum in the Mo(V) alloxanthine signal-giving species.

Mechanism of interaction of substrates with the molybdenum centre of xanthine oxidase in the catalytic reaction

Our results are subject to the normal limitation of e.x.a.f.s. (cf. Scott, 1984) and, as indicated above, not every ligand is necessarily detected. However, the method is at its most reliable when applied to oxo ligands of molybdenum (cf. Flood, 1986). Results are thus particularly pertinent to understanding the catalytic mechanism of xanthine oxidase, in which an oxo ligand has been proposed to be involved. In Scheme 1, the structures of the Inhibited species and the alloxanthine complex are compared with two alternative structures put forward by George & Bray (1988) for the Very Rapid species, which is a transient Mo(V) intermediate detected with some substrates during the catalytic reaction. These workers analysed earlier work (Tanner *et al.*, 1978; Gutteridge & Bray, 1980; Malthouse *et al.*, 1981*b*) on ligand hyperfine couplings in the Very Rapid species (as summarized in Schemes 1*d* and *e*). They also evaluated the nuclear quadrupole coupling tensor, as well as the hyperfine coupling tensor, for ^{97}Mo , concluding that the Very Rapid species must bear both an axial oxo and an equatorial sulphido ligand, as shown in each of the alternative structures (Schemes 1*d* and *e*). Thus our results on the Inhibited species and on the alloxanthine complex, following on from the work of George & Bray (1988), serve to add further emphasis to the idea that the oxo group may be retained in most, if not all, reduced xanthine oxidase species.

Scheme 2(a) shows the catalytic reaction for oxidation by xanthine oxidase of a substrate molecule, RH, as put forward in earlier work from this laboratory (see Bray, 1988). Scheme 2(b) shows an alternative scheme, evidence for the feasibility of which comes from calculations *ab initio* (Rappé & Goddard, 1982) related to molybdenum catalysts. Both schemes are compatible with evidence from e.p.r. (Gutteridge *et al.*, 1978*a*; Bray, 1988) for proton transfer from substrates to the sulphido group. Distinguishing between the structures of Scheme 1(*d*) and 1(*e*) for the Very Rapid species ought to be helpful in choosing between the two reaction mechanisms. Thus the structure of Scheme 1(*d*) would be compatible with the mechanism of Scheme 2(a) if its oxo group arose by oxidation following attack on the intermediate of Scheme 2(a) by water or OH^- ion. Conversely, the alternative structure (Scheme 1*e*) cannot obviously be reconciled with Scheme 2(a). Concerning the mechanism of Scheme 2(b), the nature may not be obvious of the ligand elimination reaction that would need to occur for the Mo(VI) intermediate of this Scheme to become converted into the Mo(V) Very Rapid structure of Scheme 1(*e*). Despite this, such a structure does seem more compatible with Scheme 2(b) than with Scheme 2(a).

Scheme 2. Proposed alternative mechanisms for interaction of a substrate molecule RH with the molybdenum centre of xanthine oxidase in the catalytic reaction

(a) is the scheme put forward previously from this laboratory (cf. Bray, 1988), and (b) is a possible alternative.

Unfortunately, George & Bray (1988) were not able to decide between the two structures illustrated in Scheme 1(d) and 1(e) for the Very Rapid species. Also, the Mo(V) Very Rapid species has not so far been studied by e.x.a.f.s. [Preliminary related studies by George *et al.* (1986) ought to be treated with caution, until more detailed kinetic work (cf. Davis *et al.*, 1984) has been done.] In the meantime, it is possible to adduce support for either Very Rapid structure, and hence for either mechanism, from the present results. Thus e.p.r. parameters [*g*-values, $A(^{33}\text{S})$ and absence of $A(^1\text{H})$] (Hawkes *et al.*, 1984) for the Alloxanthine signal are fully analogous to those of the Very Rapid family (Bray & George, 1985). This tends to point towards Scheme 1(e) for the Very Rapid structure, and hence to the mechanism of Scheme 2(b). However, ^{17}O data argue against this, in that coupling to this nucleus is readily detected (Gutteridge & Bray, 1980) in the Very Rapid signal, but not (Hawkes *et al.*, 1984) the Alloxanthine signal. Unless differences of oxygen exchange in the relevant species complicate such a comparison, ^{17}O data are thus inconsistent with the oxygen ligands being similarly located in the two Mo(V) species. Conversely, analogy between the structures of Scheme 1(a) and 1(d), and the clear possibilities for side reactions leading from a Very Rapid species, as in Scheme 1(d), to a stable Inhibited species, as in Scheme 1(a), tend to favour Scheme 1(d) for the Very Rapid structure, and so Scheme 2(a) for the mechanism. Nevertheless, the structure of Scheme 1(d) cannot be fully accepted for the Very Rapid species until direct evidence is forthcoming for the two oxygen ligands. If hyperfine coupling from the axial oxo ligand is so weak as to have escaped detection by e.p.r., it might nevertheless show up in future ENDOR experiments. Further work is thus required.

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REFERENCES

- Baines, J. T. M., Garner, G. D., Hasnain, S. S. & Morell, C. (1986) *Nucl. Instrum. Methods Phys. Res. Sect. A* **246**, 565–568
- Barber, M. J., Bray, R. C., Lowe, D. J. & Coughlan, M. P. (1976) *Biochem. J.* **153**, 297–307
- Bordas, J., Bray, R. C., Garner, C. D., Gutteridge, S. & Hasnain, S. S. (1980) *Biochem. J.* **191**, 499–508
- Bray, R. C. (1984) in *Biological Systems: Structure and Analysis* (Diakun, G. P. & Garner, D., eds.), pp. 1–7, S.E.R.C. Daresbury Laboratory, Warrington
- Bray, R. C. (1988) *Q. Rev. Biophys.* **21**, 299–329
- Bray, R. C. & George, G. N. (1985) *Biochem. Soc. Trans.* **13**, 560–567
- Bray, R. C. & Gutteridge, S. (1982) *Biochemistry* **21**, 5992–5999
- Bray, R. C., Barber, M. J., Lowe, D. J., Fox, R. & Cammack, R. (1975) *FEBS Symp.* **40**, 159–172
- Coughlan, M. (1980) *Molybdenum and Molybdenum-Containing Enzymes*, Pergamon Press, Oxford
- Cramer, S. P. & Hille, R. (1985) *J. Am. Chem. Soc.* **107**, 8164–8169
- Cramer, S. P., Hodgson, K. O., Gillum, W. O. & Mortenson, L. E. (1978) *J. Am. Chem. Soc.* **100**, 3398–3407
- Cramer, S. P., Wahl, R. & Rajagopalan, K. V. (1981) *J. Am. Chem. Soc.* **103**, 7721–7727
- Davis, M. D., Olson, J. S. & Palmer, G. (1984) *J. Biol. Chem.* **259**, 3526–3533
- Flood, A. (1986) Ph.D. Thesis, University of Manchester
- George, G. N. (1983) D.Phil. Thesis, University of Sussex
- George, G. N. & Bray, R. C. (1988) *Biochemistry* **27**, 3603–3609
- George, G. N., Bray, R. C. & Cramer, S. P. (1986) *Biochem. Soc. Trans.* **14**, 651–652
- George, G. N., Turner, N. A., Bray, R. C., Boxer, D. A., Morpeth, F. F. & Cramer, S. P. (1989) *Biochem. J.* **259**, 693–700

- Gurman, S. J., Binsted, N. & Ross, I. (1984) *J. Phys. C* **17**, 143–151
- Gutteridge, S. & Bray, R. C. (1980) *Biochem. J.* **189**, 615–623
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978*a*) *Biochem. J.* **175**, 869–878
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978*b*) *Biochem. J.* **175**, 887–897
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Hawkes, R. R., George, G. N. & Bray, R. C. (1984) *Biochem. J.* **218**, 961–968
- Hille, R., George, G. N., Eidsness, M. & Cramer, S. P. (1989) *Inorg. Chem.*, in the press
- Hinch, G. D., Wycoff, D. E. & Murmann, R. K. (1986) *Polyhedron* **5**, 487–495
- Joyner, R. W., Martin, K. J. & Meeham, P. (1987) *J. Phys. C* **20**, 4005–4012
- Malthouse, J. P. G., Williams, J. W. & Bray, R. C. (1981*a*) *Biochem. J.* **197**, 421–425
- Malthouse, J. P. G., George, G. N., Lowe, D. J. & Bray, R. C. (1981*b*) *Biochem. J.* **199**, 629–637
- Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
- Massey, V., Komai, H., Palmer, G. & Elion, G. B. (1970) *J. Biol. Chem.* **245**, 2837–2844
- Morpeth, F. F. & Bray, R. C. (1984) *Biochemistry* **23**, 1332–1338
- Nishino, T., Nishino, T. & Tsushima, K. (1981) *FEBS Lett.* **131**, 369–372
- Pick, F. M., McGartoll, M. A. & Bray, R. C. (1971) *Eur. J. Biochem.* **18**, 65–72
- Pinhal, N. M., Bray, R. C., Turner, N. A. & Lowe, D. J. (1989) *Proc. Int. Congr. Biochem.* 14th, Prague, in the press
- Rappé, A. K. & Goddard, W. A. (1982) *J. Am. Chem. Soc.* **104**, 3287–3294
- Scott, R. A. (1984) in *Structural and Resonance Techniques in Biological Research* (Rousseau, D. L., ed.), pp. 295–362, Academic Press, Orlando
- Spiro, T. G. (1985) *Molybdenum Enzymes*, Wiley-Interscience, New York
- Stiefel, E. I., Newton, W. E. & Pariyadath, N. (1977) in *Chemistry and Uses of Molybdenum* (Mitchell, P. C. H. & Seaman, A., eds.), pp. 265–270, Climax Molybdenum Co., London
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, 1328–1330
- Turner, N. A., Bray, R. C. & Diakun, G. (1988) *Abstr. Int. Conf. Biophysics and Synchrotron Radiation* 2nd, Chester, pp. 234–236
- Ventom, A. M., Deistung, J. & Bray, R. C. (1988) *Biochem. J.* **255**, 949–956
- Williams, J. W. & Bray, R. C. (1981) *Biochem. J.* **195**, 753–760
- Yamanouchi, K. & Enemark, J. H. (1979) *Inorg. Chem.* **18**, 1626–1633

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