The isolation of demolybdo xanthine oxidase from bovine milk

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It was deduced many years ago from indirect evidence that demolybdo xanthine oxidase is present in normal bovine milk. This has now been confirmed by isolation of this enzyme form by a method based on the folate–gel affinity-chromatography procedure described by Nishino & Tsushima [(1986) *J. Biol. Chem.* **261**, 11242–11246]. Enzymic and spectroscopic properties of demolybdo xanthine oxidase, which retains flavin and iron–sulphur centres, are generally in accordance with expectations. Like the normal enzyme, it yields on denaturation material fluorescing at 460 nm. Molybdenum cofactor activity measured by the *Neurospora crassa nit-1* assay in the presence of added molybdate was 33% of that of the normal enzyme. The absorption spectrum in the near-u.v. region differs slightly, but significantly, from that of the active and desulpho forms of the enzyme. It is concluded that the molybdenum cofactor site contains a pterin-like material not identical with that in the normal enzyme. The significance of the occurrence of demolybdo xanthine oxidase in milk is discussed, and evidence in the literature for demolybdo forms of other molybdoenzymes is briefly reviewed. Additional studies on the use of the affinity procedure for large-scale preparation of high-activity xanthine oxidase are described. In agreement with our ability to isolate the demolybdo enzyme, the procedure appears less effective in eliminating the demolybdo than the desulpho enzyme.

INTRODUCTION

Xanthine oxidase from bovine milk is the most extensively studied of the molybdoenzymes that depend on the pterin-containing molybdenum cofactor (Bray, 1975, 1988). It has long been known that purified preparations of enzymically active xanthine oxidase are almost invariably contaminated with inactive forms (Morell, 1952). One such form, now termed ‘desulpho xanthine oxidase’ has been thoroughly investigated. Like the active enzyme, it contains one FAD molecule and two [2Fe–2S] iron–sulphur clusters per subunit of M_r about 150000. However, its molybdenum centre differs from that of the active enzyme in that the metal has lost a sulphido ligand, replaced in the desulpho enzyme by an oxo ligand (Gutteridge *et al.*, 1978; Cramer *et al.*, 1981). Conversion of the active enzyme into the desulpho form is brought about by treatment with cyanide (Massey & Edmondson, 1970) or H_2O_2 (George, 1983) and results in loss of activity to all reducing substrates except NADH, which reacts at the flavin, rather than at the molybdenum, site.

A second inactive form of the enzyme, deficient in molybdenum, was postulated many years ago (Avis *et al.*, 1958; Bray *et al.*, 1961) to explain the non-stoichiometric and variable molybdenum contents of purified preparations of milk xanthine oxidase. Hart *et al.* (1970) introduced a purification procedure that involved treating the enzyme with high concentrations of sodium salicylate. This resulted in increased specific activities and molybdenum contents, which these workers (see also Bray, 1982) explained by postulating that salicylate, under the conditions used, acted as a selective denaturant for the demolybdo form of the enzyme.

Nevertheless, evidence for demolybdo xanthine oxidase in milk has remained indirect. Furthermore, apparently careful analysis of milk xanthine oxidase preparations by some workers (see, e.g., Massey *et al.*, 1969) has yielded no indications of the presence of the demolybdo enzyme in their samples.

Within the last few years, an efficient affinity-chromatography procedure has become available (Nishino *et al.*, 1981; Nishino & Tsushima, 1986) for purification of xanthine oxidase. We now describe application of this procedure to the isolation, in a state approaching homogeneity, of demolybdo xanthine oxidase from bovine milk from a normal dairy herd. Gardlik *et al.* (1987) have very recently described a closely related independent study. Previously Johnson *et al.* (1974) described the partial purification on a small scale of xanthine dehydrogenase, low in molybdenum, from the livers of tungsten-treated rats.

MATERIALS AND METHODS

Activity measurements, analyses and e.p.r. measurements on xanthine oxidase samples

Xanthine oxidase activity was assayed spectrophotometrically at 295 nm and 23.5 °C with xanthine as substrate. Activities are expressed as ‘AFR’ values, defined as $\Delta A_{295}/\text{min}$ divided by the A_{450} of the xanthine oxidase in the assay (Hart *et al.*, 1970; Bray, 1975). Concentrations of the enzyme monomer were estimated from A_{450} measurements by taking ϵ as $36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Bray, 1975). Molybdenum was estimated colorimetrically with toluene-3,4-dithiol, after wet-ashing with $HClO_4/H_2SO_4$ as described by Hart *et al.* (1970).

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To determine the molar absorption coefficient for demolybdo xanthine oxidase/mol of FAD, the flavin content was measured spectrophotometrically after denaturation of the enzyme with trichloroacetic acid, by taking $\epsilon_{450\text{nm}}$ for FAD as $11300 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The proportion of desulpho xanthine oxidase in purified enzyme samples was estimated by comparing the intensity of the Slow Mo(V) e.p.r. signal from the sample with that from the desulpho enzyme. The latter (used here and for experiments on reduction by NADH) was prepared by treatment with cyanide (Massey & Edmondson, 1970) of xanthine oxidase from procedure H1,2 (see below). Slow signals were elicited by reducing anaerobically for 20 min at 20–25 °C with 5–10 mM-dithionite (Bray & Gutteridge, 1982). Linearity, in the region of interest, of the relation (McGartoll *et al.*, 1970; Edmondson *et al.*, 1972) between intensity of this signal and the content of the desulpho enzyme was checked by making small additions of the latter to xanthine oxidase purified by procedure N2 (see below).

E.p.r. spectra at 9.3 GHz were recorded on a Varian E9 spectrometer interfaced to a computer (George & Bray, 1988). Mo(V) and FADH $\cdot$ (free radical) signals were recorded at 120 K with 10 mW microwave power and 0.16 mT modulation amplitude. For Fe-S signals, 16 K, 1 or 50 mW and 1 mT were used.

Molybdenum cofactor

Assays of the molybdenum-cofactor content of enzyme samples were carried out by using the apo-(*nit-1* nitrate reductase) assay of Hawkes & Bray (1984), after liberating the cofactor from the xanthine oxidase anaerobically with dimethyl sulphoxide (Hawkes & Bray, 1984; Turner *et al.*, 1987).

Fluorescence spectra were recorded on oxidized samples prepared by a modification of the method described by Johnson *et al.* (1984) for obtaining fluorescent Form B of the cofactor. Solutions of xanthine oxidase, demolybdo xanthine oxidase, biopterin (from Sigma), purified molybdenum cofactor (see below) and FAD (from Sigma), all 20 μM in 0.01 M-Tris/HCl buffer, pH 7.0, were adjusted to pH 2.5 with HCl, protected from light with aluminium foil and heated in a boiling-water bath for 20 min. An aliquot of each was then added to 0.05 M-ammonium acetate buffer (3 ml) to give a final concentration of 0.3 μM for biopterin and 1.0 μM for the other samples. pH values were adjusted to 10.1 ± 0.4 by adding NH_4OH . All the samples were clear, and fluorescence spectra were recorded without any treatment to remove FAD (cf. Siefermann-Harms *et al.*, 1985). A Perkin-Elmer LS-3 fluorescence spectrometer was used.

Purified active molybdenum cofactor for use in the above fluorimetric work was prepared from xanthine oxidase as follows, by working in an anaerobic glove cabinet (J. Deistung & R. C. Bray, unpublished work). Enzyme purified by procedure H2 (see below) was denatured by heating with SDS. The cofactor so liberated was then purified by chromatography on DEAE-Sephacel (Pharmacia) and by gel filtration on Sephadex G-10 (Pharmacia). Specific activity of the product in the cofactor assay, on a per-mol-of-Mo basis, was about 80% of the limiting value established by Hawkes & Bray (1984) and was essentially unchanged by addition of molybdate.

Xanthine oxidase purification by Step 1 described by Hart *et al.* (1970) (procedure H1)

The starting material for all the purification work described below was xanthine oxidase prepared from the milk of a normal dairy herd (A.F.R.C. Food Research Institute, Reading RG2 9AT, Berks., U.K., formerly the National Institute for Research in Dairying). Cream was isolated and buttermilk was prepared in the presence of salicylate and EDTA as stabilizers. After incubation with pancreatin, butanol was added and an $(\text{NH}_4)_2\text{SO}_4$ fractionation carried out, the procedure being exactly as described by Hart *et al.* (1970).

Xanthine oxidase purification by selective denaturation with sodium salicylate (procedure H2)

Xanthine oxidase prepared as described above was treated with high concentrations of sodium salicylate (Step 2a described by Hart *et al.*, 1970) under the conditions indicated by Bray (1982). The enzyme was dialysed exhaustively against 0.1 M-pyrophosphate buffer, pH 7.0 (prepared from $\text{Na}_4\text{P}_2\text{O}_7$ and HCl), containing 1 mM-EDTA, and the enzyme concentration (from A_{450}) was adjusted to 16–33 μM . Solid sodium salicylate was added to a concentration of 0.2 M and the solution was heated to 37° for 90 h. After cooling, precipitated material was removed by centrifuging for 1 h at 40000 g_{av} , and sodium salicylate was removed by gel filtration.

Xanthine oxidase purification by affinity chromatography of the free enzyme (procedure N1)

Our affinity-chromatography procedures were modelled closely on those of Nishino *et al.* (1981) and Nishino & Tsushima (1986), though minor changes were introduced. Folic acid (Fluka) was linked to AH(aminoethyl)-Sephacel 4B (from Pharmacia) by using 1-ethyl-3-(3-dimethylpropyl)carbodi-imide (Sigma) by the method of Nishino *et al.* (1981). Buffers for affinity chromatography were 0.05 M-Tris adjusted to pH 7.8 with HCl, and 0.1 M- $\text{Na}_4\text{P}_2\text{O}_7$, adjusted to pH 8.5 with acetic acid. Both buffers contained 0.2 mM-EDTA. Buffer A was 4 vol. of the Tris buffer plus 1 vol. of the pyrophosphate buffer, and Buffer B was 3.5 vol. of Tris plus 1.5 vol. of pyrophosphate. Before use, the affinity gel was equilibrated with Buffer A. After each use it was washed with 3 bed volumes of pyrophosphate buffer, then re-equilibrated with Buffer A. Columns of up to 3.5 cm inner diam. $\times$ 31 cm high were used. The gel was protected from light by aluminium foil at all stages.

Xanthine oxidase, partially purified by using Procedure H1, or H1 and H2, was equilibrated with Buffer A, by dialysis or gel filtration, then applied to the affinity column. The load was found to be quite critical, and the column sometimes behaved anomalously the first time it was used. Optimum results were generally achieved with about 30 nmol of enzyme/ml of gel, which gave a coloured zone that extended about one third of the way down the column, most of the material absorbing at 450 nm being bound to the column. The column was then washed with 4 bed volumes of Buffer B, causing the coloured band to spread almost to the bottom. A small amount of coloured material [containing demolybdo xanthine oxidase; see below] was eluted at this stage, as were colourless protein impurities and, in one case, small amounts of a material presumed to be lactoperoxidase.

Xanthine oxidase was then eluted with about 2 bed volumes of Buffer B containing 2.5 mM-allopurinol. Recovery was improved by running about 1 bed volume of this solution on to the column, then stopping the flow for 1 h before continuing elution. We found that allopurinol was as effective as hypoxanthine [used by Nishino *et al.* (1981)] in eluting the enzyme. Its use had the advantage of leaving the enzyme ready for the subsequent stage of affinity purification, as described below. Where this later stage was omitted, the enzyme was liberated from the alloxanthine complex as described in the next subsection. Additional xanthine oxidase-containing fractions could be recovered from the column during regeneration with pyrophosphate buffer, but had relatively high A_{280}/A_{450} values (e.g. 7.0 in one experiment).

Xanthine oxidase purification by affinity chromatography of the alloxanthine complex (procedure N2)

Xanthine oxidase, eluted from the affinity column during procedure N1 as the alloxanthine complex, was used directly for the next purification step. It was first concentrated to about 10–20 % of the bed volume of the affinity column by using ultrafiltration, gel-filtered into Buffer A, then immediately re-applied to the original column (or to a second similar column) equilibrated with this buffer. Xanthine oxidase having the highest activity did not bind to the column and was obtained by washing with 2 bed volumes of Buffer B. The enzyme so obtained was concentrated by ultrafiltration, then liberated from the alloxanthine complex by treatment with $K_3Fe(CN)_6$. Optimum re-activation was achieved by using 10–15 mol/mol of enzyme and incubating in the presence of 1 mM-salicylate for 24 h at 4 °C. The final product was gel-filtered into 0.05 M-Bicine, adjusted to pH 8.2 with NaOH, containing 0.2 mM EDTA. The half-time for re-activation by $K_3Fe(CN)_6$ was about 3 h in Buffer B or in pyrophosphate buffer. Bicine could not be employed, since it was found to react with $K_3Fe(CN)_6$. If the incubation period was extended, the activity of the enzyme decreased slightly. The bulk of the xanthine oxidase, which, after treatment with $K_3Fe(CN)_6$, had a lower AFR, could be eluted from the column either as a single fraction with pyrophosphate buffer or as two fractions by using Buffer B containing 2.5 mM-allopurinol, then pyrophosphate buffer.

Isolation of demolybdo xanthine oxidase

The starting material was the fraction from xanthine oxidase purified by procedure H1 that did not bind to the folate-affinity column during procedure N1. Such material, isolated from about 8 μ mol of xanthine oxidase, was concentrated by ultrafiltration and resubmitted to procedure N1 by using about 12 nmol of enzyme/ml of column bed volume. Fractions containing demolybdo xanthine oxidase, detected by absorption at 450 nm, did not bind to the affinity column and were eluted with Buffer A. Pooled fractions were dialysed against 10 mM-Tris/HCl, pH 8.2, containing 0.5 mM-cysteine hydrochloride and loaded on to a DEAE-Sephacel (Pharmacia) ion-exchange column equilibrated with the same buffer. Fractions were eluted by using a gradient of the equilibration buffer containing 0 to 400 mM-KCl. Those having the highest absorbance at 450 nm were

concentrated by ultrafiltration and chromatographed on a Sephacryl S-300 (Pharmacia) gel-filtration column, equilibrated with 50 mM- Na^+ /Bicine buffer, pH 8.2. In some cases demolybdo xanthine oxidase prepared by this procedure was further purified by chromatography on an Ultrogel hydroxyapatite column (from LKB) equilibrated with 100 mM-potassium phosphate buffer, pH 7.8, containing 1 mM-triethylenetetramine. Demolybdo xanthine oxidase was eluted by using a gradient of the same buffer to 600 mM.

RESULTS AND DISCUSSION

Isolation of demolybdo xanthine oxidase

Hart *et al.* (1970) described alternative procedures for isolating purified xanthine oxidase samples from butter-milk obtained from the milk of a normal dairy herd. As is described below, we have applied the affinity procedures of Nishino *et al.* (1981) and Nishino & Tsushima (1986) to purify such samples further. These affinity methods depend on interaction between the enzyme and folate, covalently linked to an agarose gel. Folate inhibits

Scheme 1. Idealized representation of the different dimeric species present in xanthine oxidase samples and their behaviour during chromatography on folate-affinity gel

The pairs of circles represent the two halves of the xanthine oxidase dimer. 'A' represents the active enzyme, and 'dS' and 'dM' the desulpho and demolybdo forms respectively. 'P' corresponds to protein impurities; the small black symbols (●) represents alloxanthine molecules bound to the active enzyme. Only species having a molybdenum centre (active or desulpho) bind to the column in procedure N1. Of these, only species with a desulpho centre (failing to react with alloxanthine) bind to it in N2.

xanthine oxidase by binding at the molybdenum site. Demolybdo xanthine oxidase molecules might therefore be separable by virtue of their failing to bind to a folate-affinity column. However, the situation is complicated by the dimeric nature of xanthine oxidase molecules.

The expected behaviour of xanthine oxidase preparations, consisting of different types of dimeric molecules, on purification by the procedures of Nishino and co-workers (Nishino *et al.*, 1981; Nishino & Tsushima, 1986) is indicated in Scheme 1. As the Scheme makes clear, it is particularly those dimers in which both halves of the molecule are in the demolybdo form that are expected to be separable from other types of xanthine oxidase dimers, by virtue of their failing to bind to the affinity column in the first step of the procedure. The demolybdo fraction is expected to be accompanied by protein impurities that are present in the starting material.

It was thus in accordance with expectations that we found, when submitting xanthine oxidase purified by the procedures of Hart *et al.* (1970) to the affinity procedures, that small amounts of material absorbing at 450 nm failed to bind to the column. As described in the Materials and methods section, we purified this putative demolybdo xanthine oxidase fraction, first by re-applying it to the affinity column and retaining the non-binding fraction, then by ion-exchange chromatography and by gel filtration.

Properties of demolybdo xanthine oxidase

Properties of samples, prepared as described above, were fully consistent with their being demolybdo xanthine oxidase. Some of the data are summarized in Table 1 and the absorption spectrum is presented in Fig. 1 and is discussed in detail below. The close relationship to xanthine oxidase is obvious from the spectrum. The A_{280}/A_{450} value of 5.7 (Table 1) is consistent with the samples being near to homogeneity, and the molar absorption coefficient at 450 nm ($36\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$) (determined as described in the Materials and methods section) is indistinguishable from that of the normal enzyme (Bray, 1975). Xanthine oxidase activity was, however, almost vanishingly small (AFR = 1; AFR was defined in

Fig. 1. Visible and near-u.v. absorption spectrum of demolybdo xanthine oxidase

—, Demolybdo enzyme; ----, spectrum of the normal enzyme (AFR 157), shown for comparison. The buffer was 0.05 M-Na⁺/Bicine, pH 8.2.

the Materials and methods section) and the molybdenum content of 0.06 mol/mol of xanthine oxidase, though significant, is nevertheless very low indeed. In agreement with these data, e.p.r. examination of the reduced demolybdo enzyme (results not shown) revealed only very weak signals due to Mo(V), as the Slow signal. On the other hand, strong signals from two types of iron-sulphur centres were observed that could not be distinguished from those of Fe-S I and Fe-S II of the normal enzyme. Similarly FADH[•] free-radical signals were observed and again could not be distinguished from those of the normal enzyme. Intactness of the flavin in the demolybdo enzyme was further confirmed by comparing (Fig. 2) the kinetics of its reduction by NADH, as followed by e.p.r., with corresponding data on the desulpho enzyme. As already mentioned, unlike other reducing substrates of xanthine oxidase, NADH reacts at the flavin rather than at the molybdenum site. In keeping with this, development of the FADH[•] e.p.r. signal occurred at a similar rate in the two samples. Its rather faster decay for the demolybdo form is consistent (cf. Olson *et al.*, 1974) with the lack of competition between flavin and molybdenum for reducing equivalents.

We examined the demolybdo enzyme for the presence of the molybdenum cofactor, both by using the apo-*(nit-1* nitrate reductase) assay, and fluorimetrically. Demolybdo enzyme was compared with the normal enzyme. Data from the assay are illustrated in Fig. 3. The slopes of the plots of nitrate liberated against enzyme added correspond, for normal xanthine oxidase to molybdenum cofactor activities, of 19 nmol of NO₂⁻/min per pmol of enzyme in the absence of molybdate and 20 nmol of NO₂⁻/min per pmol with added molybdate. These values are comparable with those reported by the above workers (Hawkes & Bray, 1984). Cofactor activity of the

Fig. 2. Time course of appearance of FADH[•] and Mo(V) e.p.r. signals during reduction of demolybdo and desulpho xanthine oxidase by NADH

Samples of demolybdo and desulpho xanthine oxidase, in 50 mM-Na⁺/Bicine, pH 8.2, were reduced anaerobically by adding 2 mM-NADH and incubated at approx. 20 °C. At the times indicated, they were frozen in liquid nitrogen and the intensities of the FADH[•] signal (■, demolybdo enzyme; ●, desulpho enzyme) and the Slow Mo(V) signal (▲, desulpho enzyme) were measured. Trace amounts only of the Slow signal were seen in the demolybdo enzyme sample and are not shown in the Figure. Signal intensities are given as electrons/molecule of enzyme.

Fig. 3. Molybdenum-cofactor activity measurements on normal and demolybdo xanthine oxidase

Molybdenum cofactor was liberated from samples of active (○, ●) and demolybdo xanthine oxidase (□, ■), of known molybdenum content, by anaerobic treatment with dimethyl sulphoxide. Molybdenum-cofactor activity was determined by reconstitution of nitrate reductase activity of the *nit-1* mutant of *Neurospora crassa*. The slopes of the curves of nmol of NO_2^- formed/min per pmol of enzyme (added as cofactor) correspond to the molybdenum-cofactor activities. Assays were carried out in the presence (●, ■) or absence (○, □) of molybdate (10 mM).

demolybdo enzyme, though much lower, was not vanishingly small. The value in the absence of added molybdate (Fig. 4) was 1.2 nmol of NO_2^- /min per pmol of enzyme, corresponding to 6% of the activity of the normal enzyme, a value that parallels the low content of molybdenum of the demolybdo enzyme (Table 1). Molybdate gave a notable stimulation of the cofactor activity of the demolybdo enzyme, to 6.5 nmol of NO_2^- /min per pmol of enzyme, corresponding to 33% of the activity found for the normal enzyme.

Fluorescence data are presented in Fig. 4. According to Johnson *et al.* (1984), molybdenum cofactor is liberated from molybdoenzymes and converted into a fluorescent oxidized pterin derivative, designated 'Form B', by heating the enzyme solution at pH 2.5 in air. Fluorescence of such samples is conveniently measured in alkaline solution, under which conditions fluorescence from the pterin is readily distinguishable from that of the

Fig. 4. Fluorescence spectra from demolybdo xanthine oxidase and related materials

Samples were heated at pH 2.5 in air to liberate and oxidize the cofactor, then adjusted to pH 10.1 ± 0.4 as described in the Materials and methods section. Fluorescence was excited at 370 nm. Samples were as follows: —, demolybdo xanthine oxidase (1.0 μM); ----, xanthine oxidase (1.0 μM); ·····, purified molybdenum cofactor (1.0 μM); - · - · -, FAD (1.0 μM); ·····, bioppterin (0.3 μM).

flavin (Siefertmann-Harms *et al.*, 1985). In Fig. 4, fluorescence spectra excited at 370 nm are compared for a number of identically treated samples prepared under such conditions. Normal xanthine oxidase gave rise, as expected, both to FAD fluorescence at 500–510 nm and to pterin fluorescence (Johnson *et al.*, 1984) at 450–460 nm. Intensity of the former was 60% of that of a sample of FAD examined at the same concentration, this value presumably resulting from incomplete liberation of enzyme-bound FAD under the conditions we employed. Similarly, pterin fluorescence was 50% of that obtained from a sample of partially purified molybdenum cofactor (see the Materials and methods section; concentration determined by molybdenum analysis). Again, this value is presumably due (cf. Hawkes & Bray, 1984) to incomplete liberation from the protein. Molybdenum cofactor in turn, showed under our conditions, 25% of the fluorescence of bioppterin that was used as a standard.

The demolybdo xanthine oxidase yielded, as expected, flavin fluorescence scarcely distinguishable in intensity (Fig. 4) from that of the normal enzyme. In addition it gave a marked shoulder in the fluorescence spectrum, corresponding to pterin fluorescence, with an intensity amounting to 80% of that from the normal enzyme.

Nature of the deficiency in demolybdo xanthine oxidase

The data indicate that demolybdo xanthine oxidase contains, in place of the normal molybdenum-carrying pteridine cofactor, a related pteridine-like non-molybdenum-bearing material. The residual molybdenum content of our demolybdo samples (Table 1) corresponds, from the *nit-1* assay results without added molybdate, to minor contamination with normal xanthine oxidase, or rather, from the AFR, to contamination with desulpho xanthine oxidase, which is fully active in the cofactor assay (Hawkes & Bray, 1984). The fluorescence data indicate, however, that the cofactor site is not vacant in most of the remaining 94% of the molecules that are molybdenum-free. Since added molybdate increased the molybdenum-cofactor activity in the *nit-1* assay of demolybdo enzyme to only one third of that of the normal enzyme, the site seems not to be wholly occupied by the normal pteridine.

The absorption spectrum (Fig. 1) shows interesting differences from that of the normal enzyme. Added significance to the small feature at 310 nm in the spectrum of the demolybdo enzyme is provided by the presence of a similar feature in the spectrum of demolybdo xanthine dehydrogenase. This is clearly apparent from the data of Johnson *et al.* (1974), though it was not commented on by these workers. The difference spectrum (not illustrated; cf. Fig. 1) of xanthine oxidase minus demolybdo xanthine oxidase shows a maximum at about 330 nm, with $\Delta\epsilon$ about $2000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value is much less than the absorption of dihydrobiopterin, a typical dihydropteridine which absorbs maximally at this wavelength, with ϵ $9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Similarly (Kaufmann, 1967), tetrahydrobiopterin has $\epsilon = 14000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 300 nm, a wavelength where there is also a small maximum in the difference spectrum. Rajagopalan and co-workers have concluded that the pteridine of the molybdenum cofactor in xanthine oxidase is in either the dihydro (Rajagopalan, 1987) or the tetrahydro (Kramer *et al.*, 1987) oxidation state. The relatively low intensity of the normal minus demolybdo difference spectrum in comparison with the spectra of pteridines, thus complements the assay and the fluorescence data in arguing strongly that the cofactor site is not vacant in the demolybdo enzyme. We conclude that the difference spectrum (cf. Fig. 1) results not only from differences in the pterin of the demolybo enzyme, but also from the loss of weak absorption bands due to the molybdenum (cf. Peterson *et al.*, 1986). However, we are not able further to interpret the spectrum.

In very closely related work, of which we were not aware until after this paper was submitted, Gardlik *et al.* (1987) also obtained what appears to be demolybdo xanthine oxidase from milk. However, these workers overlooked the dimeric nature of xanthine oxidase (cf. Scheme 1) and sought to distinguish their material from the demolybdo enzyme, terming it 'molybdopterin-free xanthine oxidase'. The properties of their material may not be in all respects identical with those of ours. Thus we saw no signs of the new Fe-S e.p.r. signal observed by these workers. Nevertheless, their data, like ours, point to the molybdenum cofactor site not being totally vacant in the demolybdo enzyme. Our quantitative *nit-1* cofactor assays [in contrast with the qualitative ones of Gardlik *et al.* (1987)] emphasize the close relation between the

molecule (or molecules) occupying this site and the molybdenum cofactor. On the other hand, Gardlik *et al.* (1987) provide the interesting additional information that the molecule does not contain a phosphate group.

Significance of demolybdo xanthine oxidase in milk

Precise analyses for the molybdenum contents of molybdoenzymes are rarely reported in the literature, but there is now a substantial body of evidence that demolybdo forms of a number of them, analogous to demolybdo xanthine oxidase, occur under ordinary circumstances *in vivo*, along with the normal enzymes. It is well established (see, e.g., Cramer & Steifel, 1985) that administration of, or growth on, tungsten gives rise to such demolybdo forms. However as reported previously, in the case of xanthine oxidase, the milk of normal cows sometimes has a content of the demolybdo enzyme that is higher than its content of the normal enzyme, this being indicated by Mo/FAD ratios of less than 0.5 (Hart *et al.*, 1970; Bray, 1982). Studies of individual cows over an extended period of time by the former workers indicated that the relative amounts of the two enzyme forms in the milk was determined by nutritional, rather than by genetic, factors. Note, again as reported by Hart *et al.* (1970), that milk xanthine oxidase shows no tendency whatever to lose molybdenum during purification. Similarly, so far as we are aware, there are no authenticated reports in the literature of molybdenum loss from other molybdoenzymes during purification. Nevertheless, in keeping with the proposition that demolybdo enzymes have a widespread occurrence in normal organisms, rabbit liver aldehyde oxidase, for example, was reported in apparently careful work from different laboratories, to contain 0.97 (Turner, 1988), 0.87 (Branzoli & Massey, 1974) or 0.47 (Felsted *et al.*, 1973) g-atom of Mo/mol. Similarly, the molybdenum contents of dissimilatory nitrate reductases isolated in different laboratories from six different micro-organisms show considerable variation (Adams & Mortenson, 1985), values reported varying from 0.2 to 1 g-atom/mol. The average of the values tabulated by these reviewers is 0.67 g-atom/mol, but they oversimplify the situation by concluding that "... all the enzymes contain approximately 1 atom of molybdenum per mole of protein."

Taken together, the data clearly imply that formation of demolybdo forms of a number of enzymes takes place under normal conditions *in vivo*. Presumably, and the data clearly points to this for xanthine oxidase, incorporation of the molybdenum cofactor is the last stage in biosynthesis of the enzymes, other stages, such as incorporation of iron-sulphur centres and of flavin, being able to proceed even when this stage is limited by some deficiency, with apparently a 'wrong' pteridine able to become incorporated. Alternatively, it might be envisaged that the correct pteridine becomes incorporated, but, in the event of a failure of the molybdenum-incorporation process, undergoes some form of degradation while remaining bound to the enzyme.

Preparation of high-activity xanthine oxidase by affinity chromatography

As indicated above (Scheme 1), Nishino *et al.* (1981) specified the use of two stages of affinity chromatography for the preparation of very highly active xanthine oxidase. The first stage (see the Materials and methods

Table 1. Properties of xanthine oxidase samples prepared by different procedures

Data on a number of xanthine oxidase samples prepared by each of five different purification procedures is summarized together with data on the demolybdo enzyme. Errors are population S.D. values for the number of samples indicated in parentheses. Preparative procedures are described in the text. H1 corresponds to xanthine oxidase purification to Step 1 of Hart *et al.* (1970) and H1,2 to material further purified to Step 2 of these workers. N1 corresponds to purification by affinity chromatography of the free enzyme as described by Nishino *et al.* (1981), and N1,2 to such material further purified by affinity chromatography as the alloxanthine complex. AFR corresponds to activity (at 23.5 °C)/ A_{450} (Bray, 1975) and Mo/XO to mol of Mo/mol of xanthine oxidase (XO; from A_{450} , with $\epsilon = 36000 \text{ M}^{-1} \cdot \text{cm}^{-1}$); percentages of desulpho enzyme ('% Desulpho') were obtained from intensity of the Slow Mo(V) e.p.r. signal; recoveries of A_{450} are expressed relative to the starting material (H1).

Procedure	AFR ^{23.5 °C}	A_{280}/A_{450}	Mo/XO	% Desulpho	Recovery (A_{450})
H1	101 ± 16(3)*	6.2–10.1*	0.64 ± 0.06(3)*†	–	[100]
H1,2	138 ± 14(33)‡	6.0 ± 0.5(24)‡	0.97 ± 0.01(3)	24(1)	40(2)*
H1;N1	104 ± 2(2)§	4.9(2)§	–	–	30–70
H1;N1,2	157 ± 8(7)	5.0 ± 0.1(4)	0.88 ± 0.05(3)¶	7 ± 3(5)	16–20**
H1,2;N1,2	169 ± 10(2)	5.0(1)§	0.94 ± 0.04(2)¶	4(1)	7–11**
Demolybdo	1(2)	5.7 ± 0.1(2)	0.06 ± 0.03(2)	–	1–2

* Data from Hart *et al.* (1970).

† Mo/FAD determined fluorimetrically.

‡ Unpublished data for samples prepared by a number of workers in this laboratory over several years.

§ Data on samples eluted with hypoxanthine.

|| The higher recovery was obtained when elution was interrupted, as described in the Materials and methods section.

¶ The difference indicated in Mo/XO between H1;N1,2 and H1,2;N1,2 is significant at the 30 % probability level (*t* test).

** Additional material with A_{280}/A_{450} close to 5.0, but with lower AFR, could be recovered from the column after procedure N2 (see the Materials and methods section).

section; procedure N1) involved affinity chromatography of the free enzyme. The second (procedure N2) involved affinity chromatography of the enzyme as the alloxanthine complex. We used, as starting material for affinity purification, xanthine oxidase prepared from buttermilk as described by Hart *et al.* (1970) and purified either to Step 1 of these workers (referred to here as procedure H1) by using pancreatin, butanol and $(\text{NH}_4)_2\text{SO}_4$, or further purified by denaturation with sodium salicylate to Step 2a, under the conditions indicated by Bray (1982) (here called procedure H2).

Properties of a considerable number of xanthine oxidase samples purified by different combinations of the above conventional and affinity procedures are summarized in Table 1. Pure 100 % functional xanthine oxidase is expected (Bray, 1975) to have one molybdenum atom per molecule, A_{280}/A_{450} close to 5.0 and AFR^{23.5 °C} about 191. Table 1 indicates the relative effectiveness of different purification steps in eliminating protein impurities, desulpho xanthine oxidase and demolybdo xanthine oxidase. In agreement with previous work, procedure H2 is effective in eliminating demolybdo xanthine oxidase, as shown by Mo/xanthine oxidase ratios near to unity for H1,2 samples. However, it does not decrease the content of the desulpho enzyme. Conversely, procedure N1 is very effective in decreasing protein impurities (H1;N1 samples), as indicated by the A_{280}/A_{450} values. (Note that SDS/polyacrylamide-gel electrophoresis is not useful in assessing xanthine oxidase purity because of proteolysis of the enzyme.) Similarly, procedure N2 is efficient in eliminating the desulpho enzyme (H1;N1,2 samples), as shown by high AFR values and low contents of desulpho enzyme (estimated from the Slow e.p.r. signal intensity). However, in agreement with our isolation of the demolybdo xanthine oxidase as described above, and in accordance with Scheme 1, this form seems not to be fully eliminated by procedure N2.

Thus our samples approximating most closely to 100 % functional xanthine oxidase (H1,2; N1,2 samples) were obtained only when applying all four purification procedures in succession. Such material, according to our analyses, is low in protein impurities and in desulpho and demolybdo xanthine oxidase. The data indicate, even for H1,2;N1,2 samples, however, an overall purity of only about 90 %, emphasizing the great difficulties associated with work with xanthine oxidase samples of the highest purity.

These studies provide the basis for selecting the best method of producing xanthine oxidase in large quantities for different types of work. For the best enzyme, all four purification steps are needed, but, for example, for physical studies of the molybdenum centre e.g. by extended X-ray absorption fine structure ('e.x.a.f.s.'), it is important to eliminate the desulpho enzyme, but the presence or absence of demolybdo enzyme is irrelevant. Thus the H1;N1,2 procedure is appropriate, with monitoring of residual desulpho enzyme by e.p.r. Where freedom from protein impurities is the only critical factor, H1,N1 is the method of choice. Furthermore, recovered material eluted from the column after the active fraction in procedure N2 (see the ** footnote to Table 1) also has this property.

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