Expression of *Drosophila melanogaster* xanthine dehydrogenase in *Aspergillus nidulans* and some properties of the recombinant enzyme[1]

Benjamin ADAMS*[2], David J. LOWE†, Andrew T. SMITH*, Claudio SCAZZOCCHIO‡, Stephane DEMAIS‡ and Robert C. BRAY§

*School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K., †Biological Chemistry Department, John Innes Centre, Norwich NR4 7UH, U.K., ‡Institut de Génétique et Microbiologie, Université de Paris-Sud, 91405 Orsay Cedex, France and §School of Chemistry, Physics and Environmental Science, University of Sussex, Brighton BN1 9QJ, U.K.

Recent crystal structures of xanthine dehydrogenase, xanthine oxidase and related enzymes have paved the way for a detailed structural and functional analysis of these enzymes. One problem encountered when working with these proteins, especially with recombinant protein, is that the preparations tend to be heterogeneous, with only a fraction of the enzyme molecules being active. This is due to the incompleteness of post-translational modification, which for this protein is a complex, and incompletely understood, process involving incorporation of the Mo and Fe/S centres. The enzyme has been expressed previously in both *Drosophila* and insect cells using baculovirus. The insect cell system has been exploited by Iwasaki et al. [Iwasaki, Okamoto, Nishino, Mizushima and Hori (2000) J. Biochem (Tokyo) **127**, 771–778], but, for the rat enzyme, yields a complex mixture of enzyme forms, containing around 10 % of functional enzyme. The expression of *Drosophila melanogaster* xanthine dehydrogenase in *Aspergillus nidulans* is described. The purified protein has been analysed both functionally and spectroscopically. Its specific activity is indistinguishable from that of the enzyme purified from fruit flies [Doyle, Burke, Chovnick, Dutton, Whittle and Bray (1996) Eur. J. Biochem. **239**, 782–795], and it appears to be more active than recombinant xanthine dehydrogenase produced with the baculovirus system. EPR spectra of the recombinant *Drosophila* enzyme are reported, including parameters for the Fe/S centres. Only a very weak 'Fe/SIII' signal ($g_{1,2,3}$, 2.057, 1.930, 1.858) was observed, in contrast to the strong analogous signal reported for the enzyme from baculovirus. Since this signal appears to be associated with incomplete post-translational modification, this is consistent with relatively more complete cofactor incorporation in the *Aspergillus*-produced enzyme. Thus we have developed a recombinant expression system for *D. melanogaster* xanthine dehydrogenase, which can be used for the production of site-specific mutations of this enzyme.

Key words: molybdenum, protein crystallography, pterin cofactor.

INTRODUCTION

The active form of the enzyme xanthine dehydrogenase (XDH; EC 1.1.1.204) is a homodimer, each subunit containing a single molybdenum cofactor (or Moco) as well as two iron–sulphur clusters and an FAD molecule. Molybdenum-cofactor-containing enzymes form a diverse group which, in general, catalyse the transfer of an oxygen atom to or from acceptor or donor molecules. Molybdenum-cofactor-containing enzymes, when categorized by the structure of their molybdenum centres, fall into three families [1]; the xanthine oxidase (XO) family, the sulphite oxidase family and the dimethylsulphoxide reductase family. The enzymes of these three families catalyse a wide variety of redox reactions, including several that are essential in both the nitrogen and sulphur cycles.

XDH is of considerable medical interest for two reasons. First, it is implicated in gout and hyperuricaemia, and is a target for drugs used to treat these conditions [2]. Secondly, its activity has been implicated in post-ischaemic reperfusion injury such as that occurring in rheumatoid arthritis and myocardial infarction [3]. Also, as the enzyme displays a broad specificity, catalysing the hydroxylation of a variety range of substrates, it represents a challenging potential target as a commercial catalyst.

Important recent advances in structural information about xanthine oxoreductase (XOR; a collective name for XDH and XO) include the crystal structure [4] of bovine milk XOR in both its oxidase and dehydrogenase forms, following on from that of the closely related aldehyde oxidoreductase [5]. Future structure/function studies on these enzymes will be hampered by difficulties in the recombinant expression. In particular, expression is accompanied by the formation of incompletely modified enzyme forms. The complex post-translational modification reactions involved in incorporating the FAD, Mo and Fe/S centres into XOR molecules are, as yet, incompletely understood. However, it is clear that these reactions often fail to go to completion, even under ordinary *in vivo* conditions. Thus similar molecules that are in various ways incomplete often accompany the fully formed XOR molecules. The former are either non-functional, or at best incompletely functional, as enzymes. Thus incorporation of Mo into bovine milk XO (EC 1.1.3.22) is often not quite complete [6,7] and there is a much more dramatic Mo deficiency in the human milk enzyme [8,9] (the bovine and other mammalian XO enzymes, but not the *Drosophila* enzyme, are formed from XDH, either reversibly, by oxidation of cysteine residues, or irreversibly, by proteolysis [3]). The desulpho-enzyme form [10] results from incomplete incorporation of the sulphido ligand of Mo, a reaction brought about by the product of a gene that has now been identified clearly [11,12]. It has been recognized recently that incorporation of the iron–sulphur clusters, particularly Fe/SI, into the human milk enzyme can also be incomplete ([8], and R. C. Bray, D. J. Lowe, B. Godber, R. Harrison and R. Eisenthal, unpublished work). In view of these findings, it is in no way

Abbreviations used: XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxoreductase; PMS, phenazine methosulphate.

[1] This paper is dedicated to Robert C. Bray, who died on 17 August 2001. His enduring work in the field of molybdoproteins will be remembered.

[2] To whom correspondence should be addressed (e-mail b.adams@susx.ac.uk).

surprising that recombinant preparations of these enzymes should consist of mixtures of incomplete enzyme forms, accompanying the fully formed holoenzyme molecules.

There have been several reports of the successful expression of recombinant XDH, in *Drosophila* [13], in a baculovirus-insect cell system [14,15] and in *Aspergillus* [16]. Of these, the baculovirus system has been, in the hands of Nishino and co-workers, the one exploited most extensively [14,15,17]. It does, nevertheless, yield a heterogeneous mixture of a number of different forms of the enzyme, of which apparently no more than 10% corresponds to the native functional enzyme form [15]. Recombinant expression in *Drosophila* [13] resulted in only very low levels of expression, estimated to be only 10% of the non-recombinant level, making the purification of enzyme on a worthwhile scale (see [18]) from such a system quite impractical.

The present investigations were carried out with a view to developing a recombinant expression system for *Drosophila* XDH in *Aspergillus nidulans*. Since this organism normally produces its own XDH in substantial quantities [19], absence of the host gene in a nonsense mutation should leave intact all the enzymes and cofactors required for the post-translational modification of the recombinant enzyme in order to yield functional protein. This expectation has been fulfilled in that expression of functional wild-type XDH was achieved. This system is shown to have advantages over the baculovirus one, in that it yields a highly active enzyme, indistinguishable from that purified from fruit flies, and containing a high proportion of fully functional molecules. Now that we have a recombinant system for the expression of XDH, we hope to use this to create site-specific mutations of this enzyme.

MATERIALS AND METHODS

Expression of *D. melanogaster* XDH cDNA in *A. nidulans*

Wild-type XDH cDNA was cloned into the shuttle vector pGPT-*pyrG*1 [20]. Prior to subcloning the cDNA into the vector, a linker was cloned between the *Xba*I site and the *Bgl*II site, resulting in the introduction of an *Hpa*I site, the loss of the *Bgl*II site and the introduction of a new *Bgl*II site overlapping the *Xba*I site. A 5′ region of the cDNA (up to the *Bcl*I site) was then cloned between the *Hpa*I and *Bgl*II sites in the modified vector to yield pGPT-XDH(nt). The 3′ region of the cDNA was then subcloned into pGPT-XDH(nt) using the *Bgl*II site in the centre of the cDNA and the *Xba*I site of the vector. Cloning of the *D. melanogaster* XDH cDNA into pGPT-*pyrG*1 resulted in the cDNA being flanked by the *Aspergillus awamori* glucoamylase promoter at its 5′ end and the *Aspergillus niger* glucoamylase terminator at its 3′ end. This promoter is regulated by carbon source, being activated by maltose and repressed by glucose. Thus when the construct is integrated into *Aspergillus*, switching media from glucose to maltose should result in the activation of XDH expression. This vector also contains the *A. nidulans pyrG* gene.

Vectors were transfected into *A. nidulans* [*pabaA1 hxA5 pyrG87*, where *hxA5* is CRM^- (no cross-reacting material; it does not contain protein that cross-reacts with antibodies to XDH) with a stop codon at residue 396] as described in [21] and selected for uracil- and uridine-independent growth. The ability to grow on hypoxanthine as a nitrogen source was used to test for the production of functional XDH. Transformants expressing XDH from the *glaA* promoter should be able to use hypoxanthine in the presence of maltose but not in its absence. Therefore, transformants were tested for their ability to grow using hypoxanthine as a nitrogen source, with maltose, but not glucose, as their carbon source.

Production and purification of recombinant XDH

To produce recombinant XDH in *Aspergillus*, 1 litre portions of minimal medium [22] were inoculated with approx. 10^9 spores and shaken at 25 °C in flasks. After 20 h the medium was changed to modified minimal medium in which the glucose was replaced by maltose. This was done by filtering the mycelium through sterile muslin, washing it with the new medium and then adding it to a clean flask containing the new medium. After a further 20 h at 25 °C the mycelium was harvested by filtering through muslin, washed with water, frozen in liquid nitrogen and stored at −80 °C.

To isolate recombinant XDH, frozen mycelium from 16 litres of culture was ground in liquid nitrogen, in batches of approx. 100 g, using an MSE Atomix blender. The resulting powder was mixed 1:1 (w/v) with 50 mM borax/1 mM EDTA, pH 9.2, containing Sigma protease-inhibitor cocktail (Sigma product number P 8215), and the resultant suspension was spun at 10000 ***g*** at 4 °C for 30 min. The supernatant was then purified by ammonium sulphate precipitation; XDH activity precipitating between 30 and 60% saturation. The ammonium sulphate pellet was washed with ammonium sulphate at 60% saturation and dissolved in 20 mM Tris/HCl/1 mM EDTA, pH 8.0. The sample was then dialysed against 20 mM Tris/HCl/1 mM EDTA/20% saturated ammonium sulphate, pH 8.0, prior to further purification, by hydrophobic affinity chromatography on phenyl-Sepharose. The activity eluted at about 5% saturated ammonium sulphate. Active fractions were pooled, dialysed against 20 mM Tris/HCl/1 mM EDTA, pH 8.0, and further purified by ion exchange on a MonoQ column, where the activity eluted at approx. 300 mM NaCl in the same buffer. Active fractions were concentrated on a Centricon concentrator and gel filtered into 50 mM Tris/HCl/1 mM EDTA, pH 8.0, frozen as beads in liquid nitrogen and stored at −80 °C or below.

Bovine milk XO

Bovine milk XO was a commercial sample (type XO2; Biozyme Laboratories, Blaenavon, Pontypool, U.K.). This had an A_{280}/A_{450} value of 5.5–5.7 and SDS/PAGE showed that the strongest band was at an M_r of approx. 150000, with perhaps 80% of the XO in a non-proteolysed state. Colorimetric molybdenum analysis and XO activity measurements [6] indicated that it consisted of approx. 50% functional enzyme (XO-a), 37% de-molybdo-enzyme and 13% desulpho-enzyme.

Assays and UV/visible spectra of XDH samples

Both assays and spectra were recorded using a Perkin Elmer Lambda 16 spectrometer. Unless otherwise stated, assays were performed in 50 mM Tris/HCl/1 mM EDTA, pH 8.0, at 23.5 °C in a final volume of 1 ml. For assays, substrate concentrations, wavelengths and absorption coefficients were: xanthine: NAD^+ assay, 100 μM xanthine, 0.33 mM NAD^+, 340 nm, $\epsilon = 6.2\ mM^{-1} \cdot cm^{-1}$; xanthine: phenazine methosulphate (PMS)/cytochrome *c* assay, 100 μM xanthine, 44 μM PMS, 13 μM cytochrome *c*, 550 nm, $\Delta\epsilon = 19.6\ mM^{-1} \cdot cm^{-1}$; acetaldehyde: NAD^+ assay, 100 mM acetaldehyde, 0.33 mM NAD^+, 340 nm, $\epsilon = 6.2\ mM^{-1} \cdot cm^{-1}$. Where there was a reaction in the absence of enzyme, its rate was subtracted from that measured in its presence. Spectra were generally recorded in Na^+-Bicine buffer. XOR subunit concentrations, for both the recombinant and the bovine enzymes, were estimated from A_{450} assuming an ϵ value of $36\ mM^{-1} \cdot cm^{-1}$ [10]. Deconvolution analysis of spectra, usually from 300 to 650 nm, was performed as described previously [23], with a routine that optimized their simulation as the sum of

contributions from reference spectra and from light scattering [as $1/(\lambda^4)$] and a horizontal baseline shift. The reference spectra used were high-purity bovine milk XO [6] and free FAD.

Extent of reduction of the enzyme by xanthine

The fraction of the enzyme reduced rapidly by xanthine, calculated as $[\Delta(A_{450}-A_{650})_{xanthine}]/[\Delta(A_{450}-A_{650})_{dithionite}]$, was determined under the following conditions. Samples were in 50 mM Tris/HCl/1 mM EDTA at 20–25 °C and additions were made anaerobically, using a glove box. The spectrum was recorded for the enzyme alone, 3 min after addition of xanthine (0.2 mM final concentration) and again 15 min after the further addition of $Na_2S_2O_4$ (1 mM final concentration).

EPR samples and measurements

Enzyme samples for EPR spectroscopy, in 50 mM Na^+-Bicine/1 mM EDTA, pH 8.2, were reduced anaerobically under appropriate conditions, then frozen in liquid nitrogen. The Rapid Mo(V) signal was generated by reduction with purine (final concentration, 10 mM) for 10 min at 5 °C. The Slow signal was generated by thawing the same sample anaerobically, adding $Na_2S_2O_4$ (final concentration, 5 mM) and incubating for 30 min at 25 °C. Fe/S signals were recorded on the same sample without further additions. Spectra were recorded at 9 GHz on a Bruker ESP300 instrument, with an NMR Gaussmeter and a microwave frequency counter. Spectra were manipulated and simulated as described previously [24]. Cu^{2+}-EDTA was used as the integration standard.

RESULTS

Expression of *D. melanogaster* XDH in *A. nidulans*

A. nidulans colonies that had been transformed with pGPT-*pyrG*1 XDH cDNA were selected for their ability to grow in the absence of uracil and uridine. In a typical transformation using 5–10 μg of plasmid DNA, 10–50 uracil-independent colonies were obtained. Transformants showing uracil-independent growth were tested for their ability to grow on plates using hypoxanthine as a nitrogen source and either glucose or maltose as a carbon sauce. Approximately half the uracil-independent colonies isolated were shown to grow on hypoxanthine in the presence of maltose, but were unable to grow on hypoxanthine in the absence of maltose, whereas all *A. nidulans* colonies transformed with pGPT-*pyrG*1 were unable to grow on hypoxanthine. This maltose-dependent complementation of the *hxA*5 phenotype shows that these clones express active XDH under the control of the *glaA* promoter. The other colonies were unable to grow on hypoxanthine under either condition. In these colonies the plasmid DNA had presumably integrated in such a way as to disrupt either the XDH cDNA or the *glaA* promoter.

Activity of recombinant XDH in crude extracts and purification of recombinant enzyme

Extracts from *A. nidulans* transformants expressing recombinant XDH were made, as described in the Materials and methods section, in 50 mM Tris/HCl/1 mM EDTA, pH 8.0. Activity was detected in these extracts in both the xanthine : PMS/cytochrome *c* and the xanthine : NAD^+ assays [16]. However, the activity was very unstable; in some extracts the half-life for the loss of activity at 0 °C in the xanthine : PMS/cytochrome *c* assay was as short as 18 min. Also, the levels of activity were not reproducible, being in some preparations close to zero. The reason for the very low stability in crude extracts is not understood. However, it may result from the conditions used for the induction. To induce biosynthesis of the recombinant protein the glucose was removed from the media and replaced with maltose. Maltose is a poor carbon source for *A. nidulans* and this change must cause complex alterations in its physiology. These changes may involve the production of many degradative enzymes, required by *Aspergillus* to recycle its biomass. Though protease inhibitors were present in the extraction buffer, it is not certain that these would have been fully effective.

It was found that if the pH of the extraction buffer was raised to 9.0 the XDH activity was more stable, while in extracts made using 50 mM borax/1 mM EDTA, pH 9.2, as the extraction buffer, activity was very much more stable, with a half-life at 0–4 °C of 10–15 h. Thus 50 mM borax/1 mM EDTA, pH 9.2, was used routinely during extraction. The mechanism by which this buffer stabilizes XDH activity was not investigated; however, there is evidence that borate binds to XOR. It has been shown to be a weak competitive inhibitor of bovine milk XO [25], and a borate-specific Mo(V) EPR signal has been described [26].

Despite a considerable effort, chromatographic conditions for the successful purification of XDH in 50 mM borax were not found. In this buffer the XDH activity did not bind to either Mono Q or phenyl-Sepharose. However, after ammonium sulphate precipitation, the XDH activity was found to be reasonably stable in 50 mM Tris/HCl/1 mM EDTA, pH 8.0. Presumably, the constituents of the extracts that destabilize the XDH are removed substantially during ammonium sulphate precipitation. To minimize losses, the purification was performed rapidly, with all column chromatography and dialysis being carried out in a cold room at 4–10 °C, with the sample kept on ice between steps. Several batches of XDH were purified. The overall yields of the purification were in the region of 5 %, with up to approx. 1 mg of XDH being obtained from 16 litres of culture.

On PAGE in the presence of SDS, the typical batches of purified protein showed one major band of M_r 120000, with minor contaminants, all of lower M_r, making up approx. 50 % of the total protein. This conclusion is supported by the A_{280}/A_{450} values, which were typically around 10; the value for the sample whose properties are reported in Table 2 (see below) was 12. As pure molybdenum-containing hydroxylases without Fe/S deficiency are expected to have an A_{280}/A_{450} of 5, [10] this is consistent with approx. 50 % of the protein in the preparations being XDH.

UV/visible spectra of XDH samples

The UV/visible spectra of samples of recombinant XDH are shown in Figure 1, in comparison with bovine milk XO [6] and wild-type XDH purified [18] from the fruit fly *D. melanogaster*. Though all the spectra are similar to one another, subtle differences were revealed by deconvolution analysis (see the Materials and methods section). These were apparent particularly between the bovine and *Drosophila* enzymes, whether recombinant or from fruit flies. The difference spectra calculated relative to the bovine milk enzyme in Figure 1 all show the same characteristic features in the 380–500 nm region. These no doubt relate to differences in the environments of the iron–sulphur centres between the enzymes from the two sources, as reflected also in the relevant EPR parameters (see below). The difference spectra shown are in fact the residuals from deconvolutions that allowed, in addition to the apparent contribution of the milk enzyme to the spectrum, for possible differences in light scattering and FAD content between the samples. As is justified elsewhere ([8], and R. C. Bray, D. J. Lowe, B. Godber,

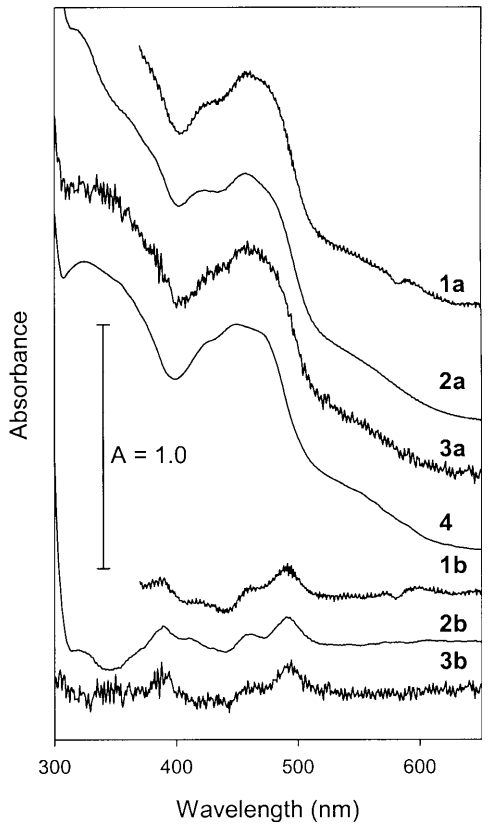

Figure 1 UV/visible spectra of recombinant *D. melanogaster* XDH samples and comparison with those of the fruit fly and bovine milk enzymes

The curves show the enzyme from the following sources: 1a, recombinant, sample 1; 2a, recombinant, sample 2; 3a, fruit fly (data from [13]); 4, bovine milk (data from [6]). All spectra have been scaled to $A_{450} = 1$ and displaced vertically as appropriate. The lower curves (1b–3b) show the corresponding difference spectra relative to the bovine milk enzyme, calculated as residuals from spectral deconvolution performed as described in the text.

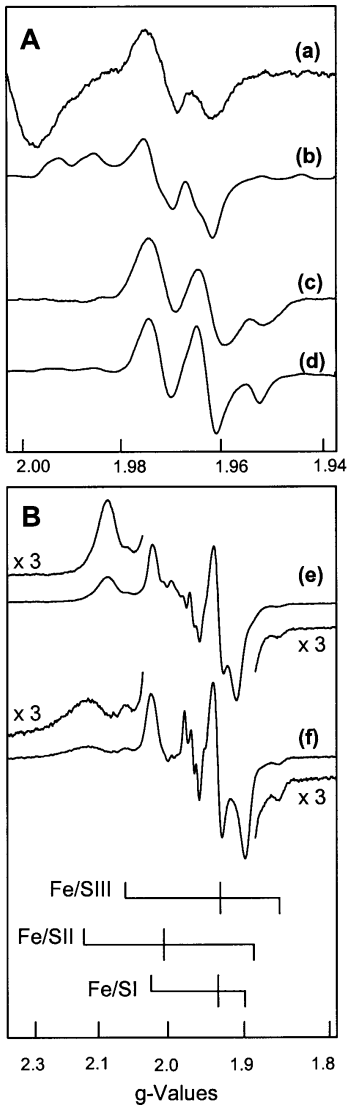

Figure 2 Comparison of EPR spectra from recombinant *D. melanogaster* XDH (traces a, c and e) and bovine milk XO (traces b, d and f), showing Mo(V) (A) and Fe/S (B) signals

Reduction was with purine as described in the Materials and methods section, giving the Rapid signal (traces a and b), followed by addition of $Na_2S_2O_4$ for the Slow signal (traces c and d), as well as for the Fe/S signals (traces e and f). The stick diagrams in (**B**) indicate, for the bovine enzyme, the g_1, g_2 and g_3 positions for Fe/SI, Fe/SII and Fe/SIII, respectively (data from Table 1). (Note that the g_2 and g_3 features of Fe/SII are scarcely detectable in the spectra shown.) Recording conditions for traces in (**A**) were: temperature, 150 K; microwave frequency, 9.3 GHz; microwave power, 10 mW; modulation amplitude, 0.19 mT in trace a and 0.39 mT in traces b–d. For those in (**B**): temperature, 20 K in trace e and 15 K in trace f; microwave frequency, 9.6 GHz; microwave power, 2 mW in trace e and 0.2 mW in trace f; modulation amplitude, 1.0 mT. In traces e and f, high- and low-field regions are shown additionally, with a 3-fold expanded vertical scale.

R. Harrison and R. Eisenthal, unpublished work), the presence of the latter is an indicator of incomplete incorporation of iron–sulphur centres into the enzyme, a feature that may be detected in other ways, e.g. by measurement [15] of the A_{450}/A_{550} absorption ratio. Deconvolution analysis (results not shown) revealed significant iron–sulphur deficiency in the recombinant sample 1 but not in sample 2 or in the fruit fly sample.

EPR spectra of the recombinant enzyme

In Figure 2, Mo(V) (Figure 2A) and Fe/S (Figure 2B) spectra from a recombinant XDH sample are compared with the corresponding spectra from the bovine milk enzyme. For the bovine enzyme, reduction with purine gave the Rapid signal from the functional enzyme, as expected (Figure 2A, trace b), while reduction with $Na_2S_2O_4$ gave the Slow signal from the desulpho-enzyme (Figure 2A, trace d; the line shape of this signal is slightly different from the normal one [27] due to the presence of purine [28]). The recombinant enzyme also gave a signal that, though slightly different from that for the bovine enzyme, is clearly of the Slow form (Figure 2A, trace c). Also, there was a recognizable Rapid signal from the recombinant enzyme, accompanied in this case by an FADH free-radical signal centred just above $g = 2.00$ (Figure 2, trace a). Integrations indicated that conversion of the enzymes into the Mo(V) state in Figure 2(A), traces a–d, was approx. 3, 20, 5 and 7 %, respectively, assuming the absence of demolybdo enzyme.

At liquid helium temperatures, the bovine enzyme sample showed not only the well-known Fe/SI and Fe/SII signals but also smaller amounts of another signal (Figure 2B, trace f), first described by Rajagopalan and co-workers [29], that we term Fe/SIII [8]. For the recombinant enzyme, all three signals are also clearly detectable (Figure 2B, trace e). However, there

Table 1 *g* values of the different Fe/S EPR signals from recombinant XDH and the bovine milk enzyme

Parameters were deduced with the help of computer simulations from the spectra of Figure 2 and from additional data. Estimated errors (S.D.) are indicated. *Dm*, *D. melanogaster*.

Enzyme	Signal	g_1	g_2	g_3
Dm	Fe/SI	2.020 ± 0.001	1.933 ± 0.001	1.910 ± 0.001
Bovine	Fe/SI	2.023 ± 0.001	1.933 ± 0.001	1.899 ± 0.001
Dm	Fe/SII	2.087 ± 0.002	1.988 ± 0.005	1.912 ± 0.005
Bovine	Fe/SII	2.122 ± 0.005	2.005 ± 0.003	1.888 ± 0.005
Dm	Fe/SIII	2.057 ± 0.001	$1.930 \pm ?$	1.858 ± 0.001
Bovine	Fe/SIII	2.060 ± 0.001	$1.930 \pm ?$	1.857 ± 0.001

Table 2 Steady-state kinetic and other parameters for samples of recombinant and endogenous *D. melanogaster* XDH

Data for XDH purified from *D. melanogaster* are taken from Doyle et al. [18]. Xanthine:PMS/cytochrome *c* (cyt *c*), xanthine:NAD^+ and acetaldehyde:NAD^+ assays were performed in triplicate under standard conditions, as described in the text. Errors are S.D. The activities of the enzymes in the xanthine:PMS/cytochrome *c* assay are given as catalytic-centre activity, in s^{-1}. The activity in other assays is given as a percentage of the activity of the same sample in the xanthine:PMS/cytochrome *c* assay. To determine K_m the xanthine:NAD^+ assay was performed in triplicate, under standard conditions, with the xanthine concentration varied between 5 and 300 μM. K_m values were determined by fitting the data to the Michaelis–Menten equation using SigmaPlot, the error given being the S.E. of the fit. The spectra of the enzyme sample are reproduced in Figure 1 (sample 1). The extent of bleaching by xanthine is expressed as $[\Delta(A_{450}-A_{650})_{xanthine}]/[\Delta(A_{450}-A_{650})_{dithionite}]$.

	XDH purified from *Drosophila*	Recombinant wild-type XDH
Reference	[18]	Present study
Xanthine:PMS/cyt *c*	32 ± 3.0 s^{-1}	31 ± 3 s^{-1}
Xanthine:NAD^+	$77 \pm 5\%$	$64 \pm 2\%$
Acetaldehyde:NAD^+	–	$37 \pm 2\%$
K_m (xanthine)	29 ± 6 μM	32 ± 2 μM
Bleaching by xanthine	–	$40 \pm 5\%$

are considerable shifts (Table 1) in some of the *g* values compared with those of the bovine enzyme, e.g. for g_3 of Fe/SI and for g_1 of Fe/SII. For the latter feature, there is also a marked (approx. 2-fold) decrease in the linewidth. *g* values of both signals are, however, within the range [30] shown by other enzymes of the XO family. Finally, it is particularly significant that Fe/SIII signals from the recombinant enzyme, though just detectable, are extremely weak in comparison with Fe/SI and Fe/SII. An attempt to evaluate the intensity of the Fe/SIII signal relative to Fe/SI, from published data, shows that this signal decreases in the order: bovine demolybdo enzyme [29] $\approx$ recombinant demolybdo dimer [14] $>$ human milk enzyme ([8], and R. C. Bray, D. J. Lowe, B. Godber, R. Harrison and R. Eisenthal, unpublished work) $\gg$ commercial bovine milk enzyme (the present study) $>$ recombinant enzyme (the present study).

Steady-state activity of purified recombinant XDH samples

Data summarizing the activity of a highly active batch of the recombinant enzyme are compared with published data for the purified endogenous fruit fly enzyme [18] in Table 2. The recombinant enzyme sample has the same activity as the enzyme purified from *Drosophila* in the xanthine:PMS/cytochrome *c* assay and marginally less in the xanthine:NAD^+ assay. In other batches the specific activity of the recombinant enzyme in the xanthine:PMS/cytochrome *c* assay was generally lower, e.g. 30 % of the values for the enzyme purified from fruit flies [18]. Such variability is no doubt due to variations in the proportion of functional enzyme in the samples, as considered in the Discussion.

The K_m value for xanthine of the recombinant protein of 32 ± 2 μM is in agreement with the value of 29 ± 6 μM reported for the endogenous protein [18].

Extent of reduction by xanthine

The extent to which the XDH sample used for the activity measurements was bleached immediately following addition of xanthine, as measured by $[\Delta(A_{450}-A_{650})_{xanthine}]/[\Delta(A_{450}-A_{650})_{dithionite}]$, is shown in Table 2.

DISCUSSION

Recombinant expression of XDH in *A. nidulans*

Clearly *A. nidulans* is able to express functional recombinant *D. melanogaster* XDH, as is shown by the complementation of the *hxA*5 phenotype by the *D. melanogaster* enzyme. This is confirmed by the enzymic activity seen in lysates. The purified protein is about 50 % pure, both from SDS/PAGE and from its A_{280}/A_{550} ratio, impurities being a complex mixture of lower-M_r proteins. Thus we have an expression and isolation system for XDH, which yields functional recombinant XDH in amounts and of a purity fully sufficient for enzymic analysis and some spectroscopic work. We have obtained, for the first time, reliable (see [31,32]) EPR data on the uncontaminated *Drosophila* enzyme, including parameters for the iron–sulphur and the molybdenum centres. However the expression system is not ideal, with induced levels of activity being variable, and with the activity being unstable in crude extracts. While instability has been alleviated partially by including borate in the extraction buffer, large losses are encountered during the isolation of the enzyme.

Recombinant XDH and 'incomplete' non-functional enzyme forms

A potential problem with any expression system for XDH is the presence of incompletely modified enzyme forms present in the purified protein. The full analysis of the complex mixtures of the various enzyme forms that arise from incomplete post-translational modification is a not a trivial task and we have not completed it in the present investigations. We have, however, used several different criteria to assess, in at least a semi-quantitative way, the proportion of our recombinant enzyme samples that correspond to fully functional XDH molecules.

Specific-activity data on the *Drosophila* enzyme, isolated from flies under mild conditions, are available for comparison from the work of Doyle et al. [18]. These workers reported a value for the catalytic-centre activity in the xanthine:PMS/cytochrome *c* assay that is virtually identical with that which we found for the recombinant enzyme studied in most detail (Table 2). These workers suggested their enzyme was approaching 100 % functional, since its specific activity appeared high in relation to that of XORs from other sources. However, a more direct way to measure the functionality of XDH is to determine the extent to which the UV/visible spectrum of the enzyme is bleached immediately on addition of xanthine. This provides a measure of the proportion of the enzyme molecules in the functional form [33–35]. Whereas precision of the measurements of bleaching at 450 nm was limited by the amounts of enzyme available, and the experimental conditions may not have been optimal, our data (Table 2) suggest, in contrast to the activity data, that our preparation of recombinant enzyme contains only some 40 %

functional enzyme. Calculation from the data of Doyle et al. [18] indicates that the extent of bleaching by xanthine of the enzyme of these workers was similar to that reported here, implying that it also was by no means fully functional. Even so, our enzyme appears to be 40 % functional. This is more than recombinant XDH produced using baculovirus-infected insect cells, for which, prior to affinity purification, the xanthine: 2,6-dichlorophenol-indophenol (DCPIP) assay indicates [14] a specific activity of less than 10 % of that of the fully active enzyme from rat liver.

Since the visible spectrum of the enzyme is dominated by the contributions of FAD and the Fe/S centres, deficiency of the latter can be detected by deconvolution with the spectra, with FAD and fully formed bovine milk XO for references. Such a procedure is in principle more quantitative than the use of A_{450}/A_{550} ratios [14,15], and has been used recently to detect Fe/S deficiency in human milk XO ([8], and R. C. Bray, D. J. Lowe, B. Godber, R. Harrison and R. Eisenthal, unpublished work). Using this technique, such a deficiency was detectable in some of our samples, though at a lower level than in the human enzyme [8]. Significantly, no Fe/S deficiency was detectable in either our sample 2 or the fruit fly enzyme [18].

One can attempt to quantify the relative amounts of the functional and desulpho-enzymes from the intensities under standard conditions of the Rapid and Slow Mo(V) EPR signals [10,27]. Absolute quantification is scarcely meaningful, since not only is the extent of conversion into the Mo(V) state critically dependent on the precise experimental conditions, but also it is expected to vary between the XDHs from different organisms. Thus the 5-fold higher Slow/Rapid signal-intensity ratio for the recombinant enzyme in comparison with the bovine enzyme sample used is no more than suggestive of a relatively high desulpho-enzyme content for the recombinant sample examined, which was of relatively low specific activity (catalytic-centre activity was 9 s^{-1} in the xanthine:NAD^+ assay).

Finally, the presence of a characteristic Fe/S EPR signal, with approx. $g_1 = 2.06$, $g_2 = 1.93$ and $g_3 = 1.86$ (termed Fe/SIII in the present work, following [8]), is a reliable indicator of replacement of some of the normal Fe/SI by a modified centre, associated with incomplete post-translational modification [8,14]. Iwasaki et al. [14] concluded that the Fe/SIII signal is characteristic of the dimeric demolybdo-enzyme, but this interpretation needs to be qualified in the light of work from this laboratory. It seems that enzyme molecules containing the cluster giving rise to this signal are unstable to proteolysis, since the literature shows it has been observed only from non-proteolysed XOR samples [36–39]. For non-proteolysed samples, the Fe/SIII signal is clearly an indicator of the presence of incompletely formed molecules. Fe/SIII was first described by Gardlik et al. [29] from preparations of demolybdo bovine XO. As shown in the present work, the signal is also observable in the commercial non-proteolysed bovine XO. However, no such signals were reported from extensive and detailed earlier EPR investigations (e.g. [36,37]) into the bovine enzyme, nor, even more significantly, were they seen in the demolybdo-enzyme of Ventom et al. [38,39]. In contrast to Gardlik et al. [29], all the above workers not reporting the signal used proteolytic enzymes in their XO preparations. In this light, the finding that our essentially non-proteolysed recombinant enzyme showed almost vanishingly small Fe/SIII levels, contrasting with the very large amounts of this cluster form in recombinant enzyme from baculovirus [14], can only mean that post-translational modification leading to the fully functional enzyme is substantially more complete in our samples than in those from baculovirus.

Taking all the data together, it is clear that the fraction of the recombinant enzyme in the fully functional state in the present work is as high as with enzyme purified from fruit flies, and considerably higher than that achieved using the baculovirus system by Nishino et al. [15]. Nevertheless, even our best samples are not free from non-functional forms. The yield of recombinant enzyme prepared from the baculovirus system has not been stated [14,15] .Thus the *Aspergillus* system may be more suitable for preparing milligram quantities of recombinant XDH.

We thank Professor R. Huber for helpful discussions in the initial stages of the work. The work was supported by the Wellcome Trust, and by a European Union network grant under framework 5 (European Union contract HPRN-CT-1999-00084).

REFERENCES

1 Hille, R. (1996) The mononuclear molybdenum enzymes. Chem. Rev. **96**, 2757–2816
2 Elion, G. B. (1989) The purine path to chemotherapy. Science **244**, 41–47
3 Nishino, T. (1994) The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in reperfusion injury. J. Biochem. (Tokyo) **116**, 1–6
4 Enroth, C., Eger, B. T., Okamoto, K., Nishino, T., Nishino, T. and Pai, E. F. (2000) Crystal structures of bovine milk xanthine dehydrogenase and xanthine oxidase: structure-based mechanism of conversion. Proc. Natl. Acad. Sci. U.S.A. **97**, 10723–10728
5 Romão, M. J., Archer, M., Moura, I., Moura, J. J., LeGall, J., Engh, R., Schneider, M., Hof, P. and Huber, R. (1995) Crystal structure of the xanthine oxidase-related aldehyde oxido-reductase from *D. gigas*. Science **270**, 1170–1176
6 Hart, L. I., McGartoll, M. A., Chapman, H. R. and Bray, R. C. (1970) The composition of milk xanthine oxidase. Biochem. J. **116**, 851–864
7 Avis, P. G., Bergel, F. and Bray, R. C. (1956) Cellular constituents. The chemistry of xanthine oxidase. Part III. Estimates of co-factors and the catalytic activities of enzyme fractions from cow's milk. J. Chem. Soc. 1219–1226
8 Bray, R. C., Lowe, D. J., Godber, B., Harrison, R. and Eisenthal, R. (1999) Properties of xanthine oxidase from human milk: the enzyme is grossly deficient in molybdenum and substantially deficient in iron–sulphur centres. In Flavins and Flavoproteins (Ghisla, S., Kronek, P., Macheroux, P. and Sund, H., eds.), pp. 775–778, Agency for Scientific Publications Rudolf Weber, Berlin
9 Godber, B., Sanders, S., Harrison, R., Eisenthal, R. and Bray, R. C. (1997) > or = 95% of xanthine oxidase in human milk is present as the demolybdo form, lacking molybdopterin. Biochem. Soc. Trans. **25**, 519S
10 Bray, R. C. (1975) Molybdenum iron–sulfur flavin hydroxylases and related enzymes. In The Enzymes, 3rd edn, vol. 12 (Boyer, P. D., ed.), pp. 299–419, Academic Press, New York
11 Watanabe, T., Ihara, N., Itoh, T., Fujita, T. and Sugimoto, Y. (2000) Deletion mutation in *Drosophila ma-l* homologous, putative molybdopterin cofactor sulfurase gene is associated with bovine xanthinuria type II. J. Biol. Chem. **275**, 21789–21792
12 Amrani, L., Primus, J., Glatigny, A., Arcangeli, L., Scazzocchio, C. and Finnerty, V. (2000) Comparison of the sequences of the *Aspergillus nidulans hxB* and *Drosophila melanogaster ma-l* genes with nifS from *Azotobacter vinelandii* suggests a mechanism for the insertion of the terminal sulphur atom in the molybdopterin cofactor. Mol. Microbiol. **38**, 114–125
13 Doyle, W. A., Burke, J. F., Chovnick, A., Dutton, F. L., Russell, C., Whittle, J. R. and Bray, R. C. (1996). Engineering and expression in *Drosophila melanogaster* of a xanthine dehydrogenase (rosy) variant. Biochem. Soc. Trans. **24**, 31S
14 Iwasaki, T., Okamoto, K., Nishino, T., Mizushima, J. and Hori, H. (2000) Sequence motif-specific assignment of two [2Fe-2S] clusters in rat xanthine oxidoreductase studied by site-directed mutagenesis. J. Biochem. (Tokyo) **127**, 771–778
15 Nishino, T., Kashima, Y., Okamoto, K., Iwasaki, T. and Nishino, T. (1997) The monomeric form of xanthine dehydrogenase expresses in baculovirus-insect cell system. In Flavins and Flavoproteins (Stevenson, K. J., Massey, V. and Williams, C. H., eds.), pp. 843–846, University of Calgary Press, Calgary, Alberta
16 Adams, B., Smith, A. T., Doyle, W. A., Bray, R. C., Ryan, M., Harrison, R., Wolstenholme, A. J., Romão, M. J., Huber, R., Demais, S. and Scazzocchio, C. (1997). Expression of wild-type and mutated *Drosophila melanogaster* xanthine dehydrogenases in *Aspergillus nidulans*. Biochem. Soc. Trans. **25**, 520S
17 Nishino, T., Okamoto, K., Hori, H., Kounosu, A. and Nishino, T. (1999) The mechanism of conversion from xanthine dehydrogenase to oxidase of rat enzyme studied by site-directed mutagenesis. In Flavins and Flavoproteins (Ghisla, S., Kronek, P., Macheroux, P. and Sund, H., eds.), pp. 791–794, Agency for Scientific Publications Rudolf Weber, Berlin
18 Doyle, W. A., Burke, J. F., Chovnick, A., Dutton, F. L., Whittle, J. R. and Bray, R. C. (1996) Properties of xanthine dehydrogenase variants from rosy mutant strains of *Drosophila melanogaster* and their relevance to the enzyme's structure and mechanism. Eur. J. Biochem. **239**, 782–795

19 Glatigny, A. and Scazzocchio, C. (1995) Cloning and molecular characterization of hxa, the gene coding for the xanthine dehydrogenase (purine hydroxylase-I) of *Aspergillus nidulans*. J. Biol. Chem. **270**, 3534–3550
20 Baron, A. J., Stevens, C., Wilmot, C., Seneviratne, K. D., Blakeley, V., Dooley, D. M., Phillips, S. E., Knowles, P. F. and McPherson, M. J. (1994) Structure and mechanism of galactose oxidase. The free radical site. J. Biol. Chem. **269**, 25095–25105
21 Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A. and Davies, R. W. (1983) Transformation by integration in *Aspergillus nidulans*. Gene **26**, 205–221
22 Scazzocchio, C., Holl, F. B. and Foguelman, A. I. (1973) The genetic control of molybdoflavoproteins in *Aspergillus nidulans*. Allopurinol-resistant mutants constitutive for xanthine-dehydrogenase. Eur. J. Biochem. **36**, 428–445
23 Bray, R. C., Adams, B., Smith, A. T., Bennett, B. and Bailey, S. (2000) Reversible dissociation of thiolate ligands from molybdenum in an enzyme of the dimethyl sulfoxide reductase family. Biochemistry **39**, 11258–11269
24 Turner, N. A., Doyle, W. A., Ventom, A. M. and Bray, R. C. (1995) Properties of rabbit liver aldehyde oxidase and the relationship of the enzyme to xanthine oxidase and dehydrogenase. Eur. J. Biochem. **232**, 646–657
25 Roush, A. and Norris, E. R. (1950) The inhibition of xanthine oxidase by borates. Arch. Biochem. Biophys. **29**, 344–347
26 Malthouse, J. P., Gutteridge, S. and Bray, R. C. (1980) Rapid type 2 molybdenum(V) electron-paramagnetic resonance signals from xanthine oxidase and the structure of the active centre of the enzyme. Biochem. J. **185**, 767–770
27 Bray, R. C. (1980) EPR of molybdenum-containing enzymes. In Biological Magnetic Resonance, vol. 2 (Berliner, L. J. and Reuben, J., eds.), pp. 45–84, Plenum Press, New York
28 Pick, F. M. and Bray, R. C. (1969) Complex-formation between reduced xanthine oxidase and purine substrates demonstrated by electron paramagnetic resonance. Biochem. J. **114**, 735–742
29 Gardlik, S., Barber, M. J. and Rajagopalan, K. V. (1987) A molybdopterin-free form of xanthine oxidase. Arch. Biochem. Biophys. **259**, 363–371
30 Bray, R. C., Turner, N. A., Le Gall, J., Barata, B. A. and Moura, J. J. (1991) Information from e.p.r. spectroscopy on the iron–sulphur centres of the iron–molybdenum protein (aldehyde oxidoreductase) of *Desulfovibrio gigas*. Biochem. J. **280**, 817–820
31 Doyle, W. A. and Bray, R. C. (1994) *Drosophila* xanthine dehydrogenase variants re-visited. Biochem. J. **300**, 915–917
32 Hughes, R. K., Bennett, B. and Bray, R. C. (1994) Xanthine dehydrogenase from *Drosophila melanogaster* – purification and properties of the wild-type enzyme and of a variant lacking iron–sulfur centers (Correction). Biochemistry **33**, 83–84
33 Morell, D. B. (1952) The nature and catalytic activities of milk xanthine oxidase. Biochem. J. **51**, 657–666
34 Edmondson, D., Massey, V., Palmer, G., Beecham, L. M. and Elion, G. B. (1972) The resolution of active and inactive xanthine oxidase by affinity chromatography. J. Biol. Chem. **247**, 1597–1604
35 McGartoll, M. A., Pick, F. M., Swann, J. C. and Bray, R. C. (1970) Properties of xanthine oxidase preparations dependent on the proportions of active and inactivated enzyme. Biochim. Biophys. Acta **212**, 523–526
36 Hille, R., Hagen, W. R. and Dunham, W. R. (1985) Spectroscopic studies on the iron–sulfur centers of milk xanthine oxidase. J. Biol. Chem. **260**, 10569–10575
37 Lowe, D. J., Lynden-Bell, R. M. and Bray, R. C. (1972) Spin–spin interaction between molybdenum and one of the iron–sulphur systems of xanthine oxidase and its relevance to the enzymic mechanism. Biochem. J. **130**, 239–249
38 Ventom, A. M., Deistung, J. and Bray, R. C. (1988) The isolation of demolybdo xanthine oxidase from bovine milk. Biochem. J. **255**, 949–956
39 Ventom, A. M. (1987). Functional and non-functional forms of some molybdenum-containing hydroxylases and their interconversions. D.Phil. Thesis, University of Sussex

Received 27 July 2001/9 November 2001; accepted 30 November 2001