Xanthine Dehydrogenase from *Drosophila melanogaster*: Purification and Properties of the Wild-Type Enzyme and of a Variant Lacking Iron–Sulfur Centers†

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ABSTRACT: Xanthine dehydrogenase has been purified to homogeneity by conventional procedures from the wild-type strain of the fruit fly *Drosophila melanogaster*, as well as from a *rosy* mutant strain (E89→K, ry^{5231}) known to carry a point mutation in the iron–sulfur domain of the enzyme. The wild-type enzyme had all the specific properties that are peculiar to the molybdenum-containing hydroxylases. It had normal contents of molybdenum, the pterin molybdenum cofactor, FAD, and iron–sulfur centers. EPR studies showed its molybdenum center to be quite indistinguishable from that of milk xanthine oxidase. As isolated, only about 10% of the enzyme was present in the functional form, with most or all of the remainder as the inactive desulfo form. It is suggested that this may be present in vivo. Extensive proteolysis accompanied by the development of oxidase activity took place during isolation, but dehydrogenase activity was retained. EPR properties of the reduced iron–sulfur centers, Fe–SI and Fe–SII, in the enzyme are very similar to those of the corresponding centers in milk xanthine oxidase. The E89→K mutant enzyme variant was in all respects closely similar to the wild-type enzyme, with the exception that it lacked both of the iron–sulfur centers. This was established both by its having the absorption spectrum of a simple flavoprotein and by the complete absence of EPR signals characteristic of iron–sulfur centers in the reduced enzyme. Despite the lack of iron–sulfur centers, the mutant enzyme had xanthine:NAD^+ oxidoreductase activity indistinguishable from that of the wild-type enzyme. Stopped-flow measurements indicated that, as for the wild-type enzyme, reduction of the mutant enzyme was rate-limiting in turnover. Thus, the iron–sulfur centers appear irrelevant to the normal turnover of the wild-type enzyme with these substrates. However, activity to certain oxidizing substrates, particularly phenazine methosulfate, is abolished in the mutant enzyme variant. This is one of the first examples of deletion by genetic means of iron–sulfur centers from an iron–sulfur protein. The relevance of our findings both to the roles of iron–sulfur centers in other systems and to the nature of the oxidizing substrate for the *Drosophila* enzyme in vivo are briefly discussed.

Eukaryotic molybdenum-containing hydroxylases (Bray, 1975; Coughlan, 1980; Hille & Massey, 1985) are a group of widely distributed enzymes of low specificity involved in a variety of types of oxidative metabolism. The group comprises mainly the xanthine oxidases and dehydrogenases and the aldehyde oxidases, and the most studied member is milk xanthine oxidase. In the enzymic reaction, oxygen incorporated into the reducing substrate molecule is derived from water, not molecular oxygen. The latter can serve as an oxidizing substrate for oxidase ("O") forms of the enzymes, but for some at least of the enzymes these may be artifacts of isolation, arising from degradation of the native NAD^+-dependent dehydrogenase ("D") forms. The enzymes are α_2 dimers, having a subunit M_r of about 150 000 and with each subunit bearing one molybdenum atom, one molecule of FAD, and two nonidentical [2Fe–2S] iron–sulfur clusters. Molybdenum is the site at which reducing substrate molecules interact with the enzymes [reducing it from the molybdenum(VI) to the molybdenum(IV) state] and is present as the pterin molybdenum cofactor (Kramer et al., 1987). Electron egress from the reduced enzymes, whether to oxygen or to NAD^+, generally occurs via the flavin. This is reduced by rapid intramolecular electron-transfer processes (Olson et al., 1974) that form part of the catalytic cycle, even though the FAD molecule is located (Barber et al., 1982; Howes et al., 1991) within the molecule at some distance from the molybdenum atom. Despite intensive study [e.g., Olson et al. (1974)], the precise role of the iron–sulfur centers in the enzymic reaction remains speculative, though their involvement in the redox reactions of turnover was first established many years ago (Bray et al., 1964).

X-ray crystallographic information is not available for any molybdenum-containing hydroxylase, and structural information is limited essentially to that derived from spectroscopic studies, particularly local information about the environment of molybdenum from EPR and other methods (Bray, 1988; Turner et al., 1989). Recently, amino acid sequence information has begun to be available (Lee et al., 1987; Keith et al., 1987; Amaya et al., 1990), and comparisons (Amaya et al., 1990; Wootton et al., 1991) have permitted tentative identification of regions of the peptide chain associated with the binding of the various cofactors. In particular [cf. Hughes et al., (1992)], by analogy with plant ferredoxins (and also bacterial succinate dehydrogenase) the four cysteines between residues 43 and 73 of the *Drosophila melanogaster* enzyme are firmly associated with iron–sulfur center Fe–SI. Also, four of the eight conserved cysteines in the immediately following section, up to residue 116, presumably supply thiolate ligands to center Fe–SII. Cysteine 116 in particular may be implicated

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in the latter center from sequence similarities with ferredoxin from *Clostridium pasteuranium*.

In *D. melanogaster*, xanthine dehydrogenase is coded by the much studied *rosy* gene (Sang, 1985), expression of which affects the eye color of the flies. More than 20 *rosy* mutant strains are currently available (Gray et al., 1991), each corresponding to a known amino acid substitution at a different site in the sequence. Characterization at the molecular level of the xanthine dehydrogenase variants from these mutant strains should provide new insights into the structure and function of molybdenum-containing hydroxylases. Study of a number of the mutants by enzyme assays of gel-filtered extracts of the flies (Bray et al., 1991; Hughes et al., 1992) focused particular attention on the E89→K (ry^{5231}) mutant, in the iron–sulfur domain. This work suggested, contrary to accepted ideas, that intramolecular electron transfer between molybdenum and flavin in the enzyme may not depend on the iron–sulfur centers.

To obtain further information, it was clearly necessary to isolate the E89→K enzyme and to study its properties in comparison with the wild-type enzyme. Previous workers (Seybold, 1974; Andres, 1976; Edwards et al., 1977; Wahl et al., 1982) have isolated samples of purified xanthine dehydrogenase from wild-type *D. melanogaster* and described limited studies of its properties. However, these investigations were not full enough for proper comparison of the enzyme from this source with other molybdenum-containing hydroxylases (Bray, 1975). We now describe the isolation and detailed characterization of the wild-type enzyme, along with comparable studies of the enzyme from the E89→K mutant strain. We show that the latter lacks iron–sulfur centers, and we describe investigations bearing on the role of these in the wild-type enzyme. Results could have a wider bearing on the roles of iron–sulfur centers in other systems. A preliminary note on parts of the work has been published (Hughes et al., 1991).

Materials and Methods

Purification of Xanthine Dehydrogenase from D. melanogaster. D. melanogaster wild-type (Canton S) and ry^{5231} (Gray et al., 1991) strains were grown by conventional means as detailed by Hughes et al. (1992). Purification was carried out on 100-g batches of frozen flies, generally at 4–8 °C. Where appropriate, enzyme samples were frozen in liquid nitrogen between purification stages. A crude extract of the flies was prepared as detailed by the above workers, by using 0.1 M Tris-HCl buffer, pH 8.0 (at 4 °C), with the following additives: leupeptin (1 μM), pepstatin (1 μM), phenylmethanesulfonyl fluoride (200 μM), EDTA (100 μM), sodium salicylate (1 mM) and dithiothreitol (1 mM). (For subsequent stages, the same buffer with all additives except phenylmethanesulfonyl fluoride was used, referred to as "buffer A".) After clarification by filtration and centrifugation, ammonium sulfate (141 g) was added to the extract (850 mL). After 30 min, the mixture was centrifuged at $25000g_{max}$ for 30 min. Ammonium sulfate (129 g) was added to the supernatant (860 mL), and after 30 min the mixture was centrifuged as before. Aldehyde oxidase activity, detectable in the crude extract, had been eliminated by this stage. The pellet was resuspended in 90 mL of buffer A, and, after 20 min of agitation, the mixture was centrifuged at $10000g_{max}$ for 10 min. The supernatant was dialyzed thoroughly against buffer A.

Acetone at −60 °C (27 mL) was slowly added with gentle stirring to the dialyzed solution (82 mL), initially at 0 °C. The temperature was progressively reduced to −5 °C during the addition by cooling with dry ice/acetone. After 30 min, the sample was centrifuged at $10000g_{max}$ for 30 min at −5 °C. The pellet was discarded, and acetone (−60 °C, 40 mL) was added to the supernatant (80 mL); the final temperature was −15 °C. After 30 min, the mixture was centrifuged as before at −15 °C. The pellet was resuspended in 50 mL of buffer A, so as to dilute the acetone to 10% (v/v) or less, and the mixture was agitated for 20 min and centrifuged at $10000g_{max}$ for 10 min. The supernatant was concentrated in an Amicon ultrafiltration cell (XM50) and repeatedly diluted with buffer A to eliminate residual acetone.

The sample (48 mL) was then loaded on to a DEAE-cellulose column (2.3 × 46 cm; Whatman DE52) equilibrated with buffer A. The column was washed with the same buffer (400 mL) and then eluted with a 400-mL linear gradient of 0.1–0.4 M NaCl in buffer A. Fractions with the highest specific activity were pooled and concentrated to 10 mL by ultrafiltration (XM50). The sample was then loaded on to a Sephacryl-HR200 gel filtration column (2.0 × 40 cm; Pharmacia) and eluted with buffer A. Fractions with the highest specific activity and containing 25 mg of total protein were pooled and concentrated as before and then further concentrated to 1 mL in a Centricon-30 microconcentrator (Amicon). The sample was loaded on to an FPLC Superose-12 gel filtration column (1.0 × 30 cm; Pharmacia) and eluted with buffer A. Fractions containing xanthine dehydrogenase activity were pooled and concentrated to 2 mL and applied to an FPLC Mono-Q anion exchange column (0.5 × 5 cm; Pharmacia). Elution was with buffer A (2 mL) followed by a gradient from 0 to 0.3 M NaCl in the same buffer (8 mL) and then from 0.3 to 0.43 M NaCl (8 mL) and finally from 0.43 to 1 M NaCl (4 mL). Fractions containing xanthine dehydrogenase activity (and now showing a pale yellow color) were pooled and concentrated to 1 mL. Final purification to homogeneity was achieved by repeating this last step. Samples of the enzyme for spectroscopic work were gel-filtered as for assay (see below).

Recovery and Recycling of Xanthine Dehydrogenase Samples. To make maximum use of the limited quantities of enzyme available, samples used for spectroscopic studies were recovered and reused for subsequent measurements. This was accomplished by removing substrates or reductants by gel filtration on Sephadex G25 or by buffer exchange in Centricon-30 microconcentrators (Amicon). This had little effect on the specific activity of the enzyme.

Xanthine Dehydrogenase Assays. Samples for activity measurements were first gel-filtered on a Sephadex G25 column (1.7 × 5.6 cm; Pharmacia) into 0.1 M Tris-HCl buffer, pH 8.0 (at 20–25 °C), containing 1 mM EDTA and no other additives. For routine assays during purification of the enzyme, a fluorimetric pterin:NAD^+ oxidoreductase assay was employed at 23.5 ± 0.2 °C. Activities of purified samples were also measured in different spectrophotometric assay systems employing different substrates, as described in detail by Hughes et al. (1992). Protein determinations were carried out by the dye-binding method (Bradford, 1976), using the Bio-Rad protein assay kit (Bio-Rad Laboratories, GmbH). Milk xanthine oxidase purified by affinity chromatography [procedure H1, N1, N2 of Ventom et al. (1988)] was used as standard, taking $A^{1\%}_{1cm}$ (280 nm) = 11.7 (Bray, 1975). All enzyme activities are expressed as [nmol of substrate ($2e^-$)] min^{-1} (mg of protein)$^{-1}$.

Milk Xanthine Oxidase Samples. These were prepared by procedures described by Ventom et al. (1988), by using denaturation with sodium salicylate or folate affinity chromatography.

Other Enzyme Assays. Xanthine oxidase samples were assayed as described by Hart et al. (1970). Aldehyde oxidase activity of crude xanthine dehydrogenase samples was measured by following the reduction of DCPIP[1] at 600 nm with 0.1 mM redistilled propionaldehyde and 12.5 μM DCPIP in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, at 23.5 °C.

Ultracentrifuge Studies. Sedimentation analysis was carried out in a Spinco model E analytical ultracentrifuge, at 56000 rpm, in 50 mM sodium Bicine buffer, pH 8.2, at 20 °C, with or without the addition of 1 mM dithiothreitol. Photographs were taken at 8-min intervals with a bar angle of 10° and were analyzed by measuring the distance of the peak from the meniscus with a traveling microscope to obtain sedimentation coefficients. Five experiments with milk xanthine oxidase (0.4 or 1.0 mg/mL) gave $S_{20} = 11.2 \pm 1.1$ S (SD).

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis (Mini Protean II equipment from Bio-Rad Ltd.) was carried out with the discontinuous buffer system (4% acrylamide stacking gel, pH 6.8, and 5 or 10% acrylamide resolving gel, pH 8.8) of Laemmli (1970). M_r markers (from Sigma) were carbonic anhydrase (29000), ovalbumin (45000), bovine plasma albumin (66000), phosphorylase B (subunit, 97000), β-galactosidase (subunit, 116000), and myosin (subunit, 205000). Electrophoresis under nondenaturing conditions employed the high-pH discontinuous buffer system (4% acrylamide stacking gel, pH 6.8, and 5% acrylamide resolving gel, pH 8.8) of Davis (1964), with or without the addition of 0.15 M β-mercaptoethanol. All gels were stained for protein with 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma). Native gels were also stained for hypoxanthine:nitroblue tetrazolium oxidoreductase activity in 0.1 M Tris-HCl, pH 8.0 [cf. Fried (1966)].

Attempted Reversal of D to O Conversion. Wild-type xanthine dehydrogenase (6.7 μM) was incubated for 30 min at 37 °C with 10 mM dithiothreitol in 50 mM sodium Bicine buffer, pH 8.2 (Batelli et al., 1973), and then gel filtered and assayed. Specific activity in the xanthine:oxygen assay decreased from 375 to 356 nmol min^{-1} (mg of protein)$^{-1}$, while that in the xanthine:NAD^+ assay was unchanged at 614 ± 2 nmol min^{-1} (mg of protein)$^{-1}$. Thus, the percent oxidase activity (cf. Tables I and II) decreased by 3%.

Absorption Spectra and Bleaching by Xanthine or $Na_2S_2O_4$. Absorption spectra were recorded on a Phillips PU8720 spectrophotometer. Bleaching measurements were made in a microcell closed with a rubber seal (Subaseal). Enzyme samples (0.4 mL) were made anaerobic in the cell by alternate evacuation and flushing with purified nitrogen, by using needles passing through the seal. Xanthine or $Na_2S_2O_4$ solutions were added from syringes through the seal. Absorbance measurements were corrected for dilution by the reductant.

Analysis for Molybdenum, FAD, and Pterin Molybdenum Cofactor. 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). FAD was estimated fluorimetrically (as total flavin) after deproteinization with K_2HPO_4 (Burch, 1957). Riboflavin (Sigma) was used as standard, but FAD (Sigma; standardized from A_{450}) or milk xanthine oxidase gave essentially identical results.

Fluorescence spectra for "fluorescent form B" derived from the pterin molybdenum cofactor (Johnson et al., 1984) were recorded at pH 10.1 on samples liberated from the enzyme and oxidized by heating at pH 2.5 according to Ventom et al. (1988). A Fluorolog Series 2 spectrofluorimeter (Spex, Metuchen, NJ) was employed, and digital subtraction was used to correct for the weaker and partially overlapping fluorescence of FAD. With excitation at 370 nm, the corrected fluorescence spectrum of form B from the *Drosophila* enzyme was identical to that from milk xanthine oxidase, with maximum emission at 482 nm. [Note that the wavelength scale appears to be in error by about 20 nm in the fluorescence spectra presented by Ventom et al. (1988).] Corrected fluorescent intensity at 482 nm was assumed to be proportional to the concentration of the pterin molybdenum cofactor. We did not analyze specifically for dinucleotide forms of the cofactor, since these have been detected (Rajagopalan, 1991) only in molybdenum enzymes from prokaryotes.

Resulfuration of Wild-Type Xanthine Dehydrogenase. Purified wild-type enzyme (29 μM) was incubated anaerobically for 16 h at 4 °C with 0.2 M sodium Bicine buffer, pH 8.5, containing 1 mM Na_2S and 1 mM $Na_2S_2O_4$. The enzyme was then gel filtered in air on Sephadex G25 and assayed. It was then subjected to a second incubation, anaerobically for 16 h at 4 °C with 10 mM Na_2S, 5 mM $Na_2S_2O_4$, and 5 μM methyl viologen in the same buffer, and then gel filtered on Sephadex G25, before activity measurements were again made.

EPR Spectroscopy. Samples of the enzymes were reduced anaerobically in EPR tubes of 3-mm internal diameter and frozen in liquid nitrogen. EPR spectra were recorded at 9.4 GHz on a Bruker ESP-300 spectrometer equipped with an NMR Gaussmeter (Bruker ER 035M) and a frequency counter (Marconi Instruments, model 2440). For liquid helium cooling, an Oxford Instruments ESR900 cryostat was used. For the weakest signals, spectral accumulation for up to 6 h was used. Background signals of similar sample tubes containing water only were recorded. For alignment, background subtraction, integration, and simulation, spectra were transferred to a DEC/VAX mainframe computer. Software was as outlined by Bray and George (1985). Integrations were performed by using 2 mM Cu^{2+}–EDTA as standard, with corrections as in earlier work [e.g., Barber et al. (1976)].

Stopped-Flow Studies. Stopped-flow measurements were carried out at 23.5 °C with the equipment (model SF-55, High Tech Scientific, Salisbury, U.K.) inside an anaerobic glove box. The time course of bleaching at 450 nm by xanthine (0.1 mM final concentration) was recorded through an interfaced microcomputer. Rate constants were obtained by fitting the recorded data, over 0.3 s, to an exponential function, by using a least-squares minimization procedure.

Results and Discussion

Purification of Xanthine Dehydrogenase from Wild-Type and E89→K Mutant D. melanogaster Strains. As summarized in Table I and described in detail under Materials and Methods, we succeeded in purifying xanthine dehydrogenase from adult wild-type *D. melanogaster* by conventional procedures, in 17% overall yield. The low content of the enzyme necessitated the use of several hundred grams of flies (weighing approximately 1 mg each) for the work described below. Table I also summarizes data on the purification of the enzyme from the E89→K mutant strain. Purification proceeded indistinguishably for the two enzyme forms, except for the different A_{280}/A_{450} values for the final products. For both enzyme variants, the specific activity of the final product (Table I), measured in the pterin:NAD^+ oxidoreductase assay, was about 3 times higher than that reported by earlier workers (Seybold, 1974; Andres, 1976).[2]

[1] Abbreviations: MV, methyl viologen; MV•, methyl viologen radical; PMS, phenazine methosulfate, DCPIP, 2,6-dichlorophenol indophenol.

Table I: Purification of *Drosophila* Wild-Type and E89→K Mutant Xanthine Dehydrogenases[a]

stage[b]	sp act[c] [nmol min^{-1} (mg of protein)$^{-1}$]	total protein (mg)	yield (%)	purification (fold)	A_{280}/A_{450}	oxidase act.[d] (%)
crude extract (100 g of flies)	0.20	12200	(100)	(1)		4
	0.19	*11700*	*(100)*	*(1)*		*2*
ammonium sulfate (30 → 55% saturation)	0.81	2650	88	4		12
	0.75	*2430*	*83*	*4*		*13*
acetone [25 → 50% (v/v)]	3.38	447	62	17		24
	3.09	*424*	*59*	*16*		*36*
DEAE-cellulose (0.1 → 0.4 M NaCl)	8.96	111	41	45		40
	7.98	*96*	*35*	*42*		*51*
Sephacryl-HR200	27.6	24.7	28	138		46
	24.1	*25.8*	*28*	*127*		*58*
FPLC Superose-12[e]	70.3	7.60	22	352	41.7	48
	68.8	*7.54*	*24*	*362*	*84*	*61*
FPLC Mono-Q (NaCl gradient; two runs)	556	0.75	17	2770	5.3	49
	540	*0.85*	*21*	*2840*	*11.9*	*66*

[a] Data on the wild-type enzyme are given in normal type and on the E89→K mutant in italics. [b] The purification procedure and the assay methods are described under Materials and Methods. [c] Activity was measured in the fluorimetric pterin:NAD^+ oxidoreductase assay at 23.5 °C and pH 8.0. [d] Percentage ratio, xanthine:oxygen/xanthine:NAD^+ oxidoreductase activities. [e] For the E89→K mutant, this purification step was carried out twice.

Table II: Specific Activities in Different Assay Systems of Purified Wild-Type and E89→K Mutant Xanthine Dehydrogenases[a]

	assay								
enzyme	xanthine: NAD^+	xanthine: O_2	pterin: NAD^+	xanthine: PMS/cyt *c*	xanthine: DCPIP	NADH: PMS/cyt *c*	NADH: DCPIP	MV•: NAD	oxidase act. (%)[b]
wild-type (fresh)	617	301	556	192	340			290	49
wild-type (aged)	614	375	553	197	332	61	92	282	61
E89→K (fresh)	577	381	540	6	6			307	66
E89→K (aged)	573	412				6	80		72

[a] Activities are expressed as nanomoles of substrate ($2e^-$) min^{-1} (mg of protein)$^{-1}$ at 23.5 °C and pH 8.0. Fresh enzyme refers to that immediately after preparation and aged enzyme to that after recovery for reuse (see Materials and Methods for details). [b] Percentage ratio, xanthine:oxygen/xanthine:NAD^+ oxidoreductase activities.

Table II gives data on the specific activity of the two enzyme forms, in eight different assay systems [as used for work on gel-filtered extracts of *Drosophila* strains by Hughes et al. (1992)]. Data are presented not only for the freshly prepared enzyme but also for aged enzyme that had been recovered for reuse. Aging had little effect on the enzymes, apart from some increases in oxidase activity, discussed below. The mutant differs from the wild-type enzyme only in the loss of activity to indophenol as acceptor with xanthine (but not with NADH) as reducing substrate and to phenazine methosulfate as acceptor with either reducing substrate.

Homogeneity, M_r, and Subunit Composition. The ratio A_{280}/A_{450} has been widely used as a criterion of purity in molybdenum-containing hydroxylases, a value of 5.0 for the ratio corresponding, for milk xanthine oxidase, to the pure enzyme. Similar, or slightly higher, values have been reported for other enzymes of this group (Bray, 1975). A ratio of 5.3 for the wild-type *Drosophila* enzyme (Table I) thus suggests high purity.

Though the subunit M_r of undegraded molybdenum-containing hydroxylases is generally about 150000, proteolysis is difficult to prevent. Early work on milk xanthine oxidase [e.g., Corran (1939), Avis et al. (1956), and Hart et al. (1970)] employed ultracentrifugation as a criterion of homogeneity. A sedimentation coefficient of about 11S was observed, corresponding to M_r for the intact enzyme dimer of approximately 300000 (Bray, 1975). While SDS gel electrophoresis shows extensive proteolysis (Nagler & Vartanyan, 1976) in standard preparations of the enzyme, milk xanthine oxidase may nevertheless be prepared to give a single main band of M_r 150000 [e.g., Sullivan et al. (1982), Cheng et al. (1988), and I. Bäkström and R. C. Bray, unpublished work]. Proteolysis has little effect on M_r, however, and has not been reported to affect the spectroscopic properties of the enzyme or the catalytic properties, except in relation to conversion to the "O" form (see below). For xanthine dehydrogenase from *D. melanogaster*, a single band of M_r 150000 has been reported in denaturing gels by Andres (1976) and by Edwards et al. (1977). However, the latter workers noted some evidence for proteolytic degradation.

For our wild-type *Drosophila* enzyme samples, we observed (Figure 1A) a single peak in the ultracentrifuge, indicating high purity, though the small amount of material available limited the sensitivity of the analysis. A sedimentation coefficient of 10.4S was calculated (see Materials and Methods), in agreement with data on the milk enzyme and consistent with the expected dimer of subunits of M_r 146898 (Keith et al., 1989). That our preparations of the *Drosophila* enzyme are essentially pure is confirmed by results (Figure 1B) of electrophoresis in nondenaturing gels stained for protein. Both the wild-type and the E89→K mutant enzymes showed a single, rather broad band. [If mercaptoethanol was not added, additional slower moving bands were observed (Figure

[2] Wahl et al. (1982) claim to have obtained enzyme having a specific activity 7-fold higher than the values obtained in the present work. However, the purification procedure used by these workers is not described in detail, evidence for homogeneity of the product is not given, and the UV/visible absorption spectrum is less well resolved than that in Figure 2. On the other hand, they claim merely that their specific activity is "nearly twice" that reported by Seybold (1974). Thus, we suggest [cf. Ventom (1987)] that a calculation error has been made and that specific activities quoted by Wahl et al. (1982) should be reduced by a factor of 10, meaning that our activities are 1.4-times higher than those of these workers.

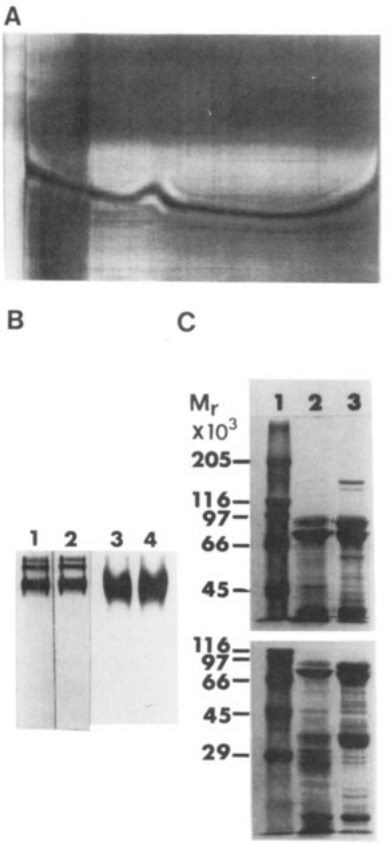

FIGURE 1: Homogeneity of purified *Drosophila* xanthine dehydrogenase preparations. (A) Sedimentation of the wild-type enzyme (0.4 mg/mL) in the ultracentrifuge (40 min at 56 000 rpm at 20 °C in the presence of dithiothreitol). (B and C) Electrophoresis in nondenaturing and SDS gels, respectively, both for the wild-type and for the E89→K enzyme variants. In panel B, lanes 1–3 correspond to wild-type and lane 4 to mutant enzyme; lanes 3 and 4 were run in the presence, and lanes 1 and 2 in the absence, of β-mercaptoethanol (0.15 M). Lanes 2–4 were stained for protein (Coomassie blue) and lane 1 for activity (see Materials and Methods). (Lane 2 corresponds to the sample of lane 1 stained additionally for protein). 10 μg samples and 5% acrylamide gels were used. In panel C the upper diagram corresponds to 5% and the lower one to 10% acrylamide gels; lanes 1–3 correspond, respectively, to M_r markers (see Materials and Methods) and the wild-type and mutant enzymes. Staining was with Coomassie blue.

1B) that stained both for protein and for xanthine dehydrogenase activity and that presumably correspond to aggregated material.][3]

On the other hand, very extensive proteolysis of our preparations, despite our use of protease inhibitors, is indicated by SDS gel electrophoresis, as shown in Figure 1C. A 150 000 M_r component is scarcely detectable for the wild-type enzyme but is definitely present in the mutant, though only as a minor component. The discrepancy, in comparison with the work of Andres (1976) and of Edwards et al. (1977), is presumably related to the purification methods employed. These workers used immunoaffinity methods, whereas we used conventional

[3] Milk xanthine oxidase behaved similarly to the *Drosophila* enzyme in nondenaturing gels, again showing evidence of aggregation in the absence of β-mercaptoethanol. Analogously, with milk xanthine oxidase, faster sedimenting material was also observed in the ultracentrifuge at pH 8.2, if dithiothreitol was not present. There is good evidence that such materials correspond to aggregated enzyme (Bray et al., 1966). Note that the sedimentation coefficient estimated for the native *Drosophila* enzyme and the value of M_r for the largest species observed in SDS gel electrophoresis (Figure 1C) are fully consistent with M_r approximately 300 000 for the xanthine dehydrogenase dimer.

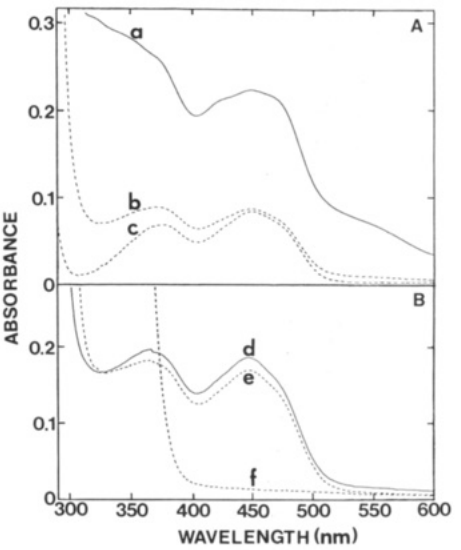

FIGURE 2: UV/Visible absorption spectra of purified *Drosophila* xanthine dehydrogenase variants: (A) oxidized enzymes in comparison with FAD; (B) reduction of the E89→K mutant enzyme. (a) Oxidized wild-type enzyme (6.2 μM) and (b) the E89→K mutant enzyme (8.2 μM). The spectrum of FAD (7.4 μM) is shown in curve c for comparison. (d) E89→K mutant enzyme in the oxidized state. (e) The same sample after reduction with xanthine (0.2 mM, 2 min reaction time). (f) Spectrum after reduction with $Na_2S_2O_4$ (0.2 mM; 2 min reaction time). The buffer was 50 mM sodium Bicine, pH 8.2.

ones. The latter are of necessity more time consuming than the former, thus giving proteases in the crude extracts more time to act before being removed. On the other hand, rather drastic conditions were required for eluting the enzyme from the immunoaffinity columns and this may be the reason for the much lower specific activities of the earlier preparations.

Extent of Conversion of Xanthine Dehydrogenase to the O Form. The conversion of D forms of molybdenum-containing hydroxylases that react with NAD^+ into O forms that react with molecular oxygen has been extensively studied (Bray, 1975; Nakamura & Yamazaki, 1982; Saito et al., 1989; Amaya et al., 1990). Though details of the changes involved remain uncertain, it is clear that proteolytic degradation and loss of –SH groups are involved. Not all molybdenum-containing hydroxylases are susceptible to D to O conversion, with enzymes from avian sources reported to be resistant. Wahl et al. (1982) claimed that the *Drosophila* enzyme does not undergo this process, though no supporting data were presented. That such a conversion, or at least a partial conversion, does in fact take place is clearly shown by the results in Table I. The ratio, initially low, of xanthine:oxygen oxidoreductase activity to that in the xanthine:NAD^+ assay, increased steadily during the purification procedure, despite precautions taken (see Materials and Methods) to minimize both proteolysis and –SH oxidation. However, in the final product from our purification, specific activity in the dehydrogenase assay remained greater than that in the oxidase assay (Tables I and II). This is in contrast to results on the milk (Bray, 1975) and rat (Waud & Rajagopalan, 1976) enzymes prepared by standard methods. That the D to O conversion was not complete at the end of the purification of the *Drosophila* enzyme is shown by the finding (Table II) that, for aged samples recovered after spectroscopic studies, the percentage of oxidase activity had increased by 12% in the case of the wild-type enzyme and by 6% for the mutant. Significantly, these increases occurred without diminution in the dehydrogenase activity. On the other hand, while, for some enzymes, some reversal of D to O conversion can be brought about (Bray, 1975) by treatment with high concentrations of dithiothreitol at elevated tem-

Table III: Analytical Data on *Drosophila* Wild-Type and E89→K Mutant Xanthine Dehydrogenases and Comparison with Milk Xanthine Oxidase[a]

parameter	*Drosophila* wild-type	*Drosophila* E89→K	milk enzyme[b]
$A^{1\%}_{1cm}$ (280 nm)[c]	12.6	8.8	11.7
A_{280}/A_{450}	5.3	11.9	5.0
ϵ_{450} (mM^{-1} cm^{-1})[d]	35	10.8	36
Mo (g·atom/mol)[e]	0.9	0.8	1
pterin Mo cofactor (mol/mol)[e,f]	1.0	0.7[g]	(1)
FAD (mol/mol)[e]	1.0	1.0	1

[a] Analytical methods are described under Materials and Methods; molar concentrations of the enzyme refer to subunit concentrations. [b] Data from Bray (1975). [c] Protein concentrations were determined by the dye-binding assay (Bradford, 1976) with milk xanthine oxidase as standard. [d] Calculated from $A^{1\%}_{1cm}$ (280 nm), A_{280}/A_{450}, and M_r 146 898 (Keith et al., 1987). [e] Enzyme concentration calculated from A_{450}. [f] Estimated fluorimetrically as "fluorescent form B" by using milk xanthine oxidase as a standard. [g] Since air oxidation is employed to obtain "fluorescent form B", it is conceivable that the rather low value obtained for the mutant enzyme may be related to its lack of iron, with the metal catalyzing the oxidation in the case of the wild-type enzyme.

peratures, we found virtually no effect of such treatment on our preparations of the *Drosophila* enzyme (see Materials and Methods).

Absorption Spectrum of the Enzyme and the Contents of FAD and Molybdenum. UV/visible absorption spectra for the *Drosophila* enzymes are profoundly different from one another, with that of the wild-type enzyme (Figure 2A, curve a) being entirely analogous to that of milk xanthine oxidase (Bray, 1975) and other molybdenum-containing hydroxylases, while the spectrum of the mutant variant (curve b) is quite similar to that of free FAD (curve c). For the wild-type enzyme, the spectrum is better resolved than that presented by Wahl et al. (1982), suggesting that the product of these workers may have been of lower purity. Specific absorption values, $A^{1\%}_{1cm}$ (280 nm) and ϵ_{450nm} for the wild-type enzyme (Table III), are scarcely distinguishable from the corresponding values for the milk enzyme. Contents of molybdenum and of FAD, determined colorimetrically and fluorimetrically, respectively, are close to 1 mol per mol of enzyme subunit, both for the wild-type and the mutant enzyme forms (Table III). So too is the content of the pterin molybdenum cofactor, estimated by a simple fluorimetric procedure following oxidation to "fluorescent form B". In earlier work on the *Drosophila* enzyme, only Andres (1976) analyzed for molybdenum and FAD, reporting much lower contents than those given in Table III.

Contamination of Functional Xanthine Dehydrogenase with Enzymically Inactive Forms. In standard preparations of milk xanthine oxidase, the functionally active enzyme is contaminated with inactive enzyme forms of two different types, demolybdo xanthine oxidase and desulfo xanthine oxidase (Bray, 1975). Both inactive forms involve changes in the molybdenum center in comparison with the functional enzyme, but not to the FAD, the Fe–S centers, or to other parts of the molecule. The demolybdo form has been isolated (Gardlik et al., 1987; Ventom et al., 1988) from milk samples and is a natural product. There are also indications of demolybdo forms of other molybdenum enzymes [see Ventom et al. (1988)]. The desulfo form of xanthine oxidase corresponds simply to replacement of a sulfido ligand of molybdenum (Mo=S) by an oxo ligand (Mo=O), a change induced by treatment with cyanide ions and in part reversed by treatment with sulfide ions. There is good evidence (Bray, 1975; Wootton et al., 1991; Turner et al., 1987) for desulfo forms of many molybdenum-containing hydroxylases. These frequently arise in vitro from degradation of the functional enzyme, but in at least one instance (Ikegami & Nishino, 1986) evidence has been presented that the desulfo form is a natural product. Incorporation of the sulfido ligand into xanthine dehydrogenase of *Drosophila* xanthine dehydrogenase in vivo appears to be controlled by the *ma-l* locus (Wahl et al., 1982; Ventom, 1987).

Specific activities and data on anaerobic bleaching of the *Drosophila* enzymes in the presence of the substrate provide evidence for the presence of large amounts of inactive enzyme in our samples, with EPR (considered in the next subsection) directly confirming that much desulfo enzyme is present. The accuracy of our molybdenum analyses (Table III) on the small amounts of the enzymes available is not sufficient to give definitive information on the content of the demolybdo enzyme, but there can be no more than relatively small amounts of this present, either in the wild-type or in the mutant enzyme.

In more detail, our specific activity values for both enzyme variants (Tables I and II) of about 0.5–0.6 μmol of substrate (mg of protein)$^{-1}$ min^{-1} at 23.5 °C are an order of magnitude lower than those of several well-studied fully functional molybdenum-containing hydroxylases (Bray, 1975; Branzoli & Massey, 1974; Cleere et al., 1974; Cleere & Coughlan, 1975; Saito & Nishino, 1989). Bearing in mind the great similarities that exist among the eukaryotic molybdenum-containing hydroxylases, this is circumstantial evidence that our preparations of the *Drosophila* enzyme contain only about 10% of the functional xanthine dehydrogenase.

Anaerobic reduction of xanthine oxidase by the substrate occurs on an extended time scale and is complicated (Swann & Bray, 1972; Olson et al., 1974). It has been established (Morell, 1952) and repeatedly confirmed [e.g., McGartoll et al. (1970) and Edmondson et al. (1972)] that when the extent of rapidly occurring anaerobic bleaching of an enzyme sample by the substrate, measured at 450 nm, is compared with the maximum bleaching obtainable in the presence of dithionite, then the percentage ratio of these quantities given the proportion of functional enzyme molecules in the sample. Application of this procedure to our preparations of the *Drosophila* enzyme confirms (Table IV) the indication from the specific activity data that our samples of both enzyme variants contain about 10% of the functional enzyme.

There is disagreement in the literature as to the extent to which the desulfo forms of molybdenum-containing hydroxylases can be caused to revert, in the presence of sulfide ions, to the functional forms. Wahl and Rajagopalan (1982) claimed to achieve 100% resulfuration, but, in this laboratory, despite considerable effort (Ventom & Bray, 1984; Ventom, 1987), no more than about 50% has been achieved under the most favorable circumstances for any molybdenum-containing hydroxylase. In earlier work on the *Drosophila* enzyme, Wahl et al. (1982) provided evidence that activity was completely lost on treatment with CN^- and could be fully restored on treatment with S^{2-}. Analogous experiments have been carried out in this laboratory (Ventom, 1987). In the present work, we endeavored to increase the activity of a preparation of the wild-type enzyme by resulfuration, successively, first under the conditions of Wahl et al. (1982) and then under those of Ventom (1987). We achieved only a modest increase in activity, with the percent functionality increasing from 11 to 15 and finally to 19.

EPR Spectra of Molybdenum(V) in the Enzyme: The Nature of the Molybdenum Center in the Drosophila Enzyme and Confirmation of the Presence of the Desulfo Enzyme. EPR spectroscopy of molybdenum-containing hydroxylases has been important in establishing the nature of the coordi-

Table IV: Anaerobic Bleaching at 450 nm of Different Enzymes by Xanthine and by $Na_2S_2O_4$: Extent and Rate Constant[a]

enzyme	AFR[b]	bleaching (%)[c] xanthine (manual)	xanthine (stopped-flow)	$Na_2S_2O_4$ (manual)	funtional enzyme (%)[d]	rate constant (s^{-1}) (xanthine)
milk xanthine oxidase[e]	22[f]	8	7	69	11	12.5 ± 2.6[g]
Drosophila xanthine dehydrogenase (wild-type)	24	8[h]	7	65[h]	12	10.2 ± 1.3[i]
Drosophila xanthine dehydrogenase (E89→K)	47	9[j]	8	96[j]	9	10.6 ± 0.3[i]

[a] All experiments were carried out in 50 mM sodium Bicine buffer, pH 8.2, at 23.5 °C. For details of procedures, see Materials and Methods. [b] For xanthine oxidase, AFR is defined (Bray, 1975) as ΔA_{295}/min at 23.5 °C in the xanthine:oxygen oxidoreductase assay, divided by A_{450} for the enzyme at the concentration used in the assay mixture. For the xanthine dehydrogenases, it is here analogously defined as ΔA_{340}/min in the xanthine:NAD^+ oxidoreductase assay, divided by A_{450}. The data indicate a limiting AFR for the fully functional dehydrogenase of 200. [c] Decrease in A_{450} measured after anaerobic addition of xanthine or $Na_2S_2O_4$. Corrections were made for dilutions; the final concentration of reductant was 0.1 mM. Measurement was made at the following approximate times after mixing: xanthine, manual mixing, 2 min; xanthine, stopped-flow, 0.3 s; $Na_2S_2O_4$, manual mixing, 2 min. [d] Calculated as 100× (average xanthine bleaching)/($Na_2S_2O_4$ bleaching). [e] Milk xanthine oxidase of similar percent functionality was investigated, as a control for the work on the *Drosophila* enzymes and in parallel with it. [f] This corresponds to 12% functional enzyme, on the basis of a limiting AFR of 190 (Bray, 1975) for the milk enzyme. [g] Because of the small amounts of *Drosophila* enzyme available and its low percent functionality, absorbance changes observed in the stopped-flow were small (ΔA about 0.006). The value given is the mean and the SD from 18 measurements of xanthine oxidase samples having a range of percent functionalities and examined at different concentrations (and including samples analogous to those from *Drosophila*). No systematic effects of concentration or of AFR on the rate constant were observed. [h] Spectral changes in the UV and visible regions were similar to those for xanthine oxidase samples of similar functionality (data not shown). [i] Mean and SD from five measurements. [j] Spectral changes in the UV and visible regions are illustrated in Figure 2.

Table V: EPR Parameters of Molybdenum(V) Species and of Reduced Iron–sulfur Centers in Wild-Type and Mutant *Drosophila* Xanthine Dehydrogenases: Comparison with Milk Xanthine Oxidase[a]

signal	enzyme	EPR parameters: parameter	1	2	3	av	ref
Rapid	average of all three[b]	g	1.9891	1.9702	1.9655	1.9750	present work
		$A(^1H)_1$	1.25	1.26	1.39	1.30	
		$A(^1H)_2$	0.46	0.32	0.15	0.31	
Rapid	milk	g	1.9893	1.9703	1.9657	1.9751	Gutteridge et al. (1978a)
		$A(^1H)_1$	1.30	1.39	1.40	1.36	
		$A(^1H)_2$	0.40	0.30	0.20	0.30	
Slow	average of all three[c]	g	1.9717	1.9670	1.9553	1.9647	present work
		$A(^1H)_1$	1.66	1.62	1.51	1.60	
		$A(^1H)_2$	0.20	0.02	0.17	0.13	
Slow	milk	g	1.9719	1.9671	1.9551	1.9647	Gutteridge et al. (1978b)
		$A(^1H)_1$	1.66	1.66	1.56	1.63	
		$A(^1H)_2$	0.16	0.16	0.16	0.16	
Fe–SI	wild-type[d]	g	2.022	1.933	1.902	1.952	present work
Fe–SI	milk	g	2.022	1.932	1.894	1.949	Hille et al. (1985)
Fe–SI	average of six[e]	g	2.021 (±0.004)	1.934 (±0.005)	1.907 (±0.011)	1.954 (±0.006)	see below[e]
Fe–SII	wild-type[d]	g	2.118	2.005	1.896	2.006	present work
Fe–SII	milk	g	2.110	1.991	1.902	2.001	Hille et al. (1985)
Fe–SII	average of six[e]	g	2.100 (±0.015)	2.001 (±0.006)	1.915 (±0.008)	2.005 (±0.004)	see below[e]

[a] Parameters given are those used in the simulated EPR spectra in Figures 3 and 4; hyperfine couplings are in mT. [b] Parameters given are the average of those used for simulations of the spectra from the *Drosophila* wild-type, E89→K, and milk enzymes (Figure 3A, curves b, d, and f, respectively). Maximum deviations in individual simulations were for g values, 0.0003; for $A(^1H)_1$, 0.10; and for $A(^1H)_2$, 0.17. Line widths were similar in the three simulations. [c] Parameters given are the average of those used for simulations of the spectra for the wild-type, E89→K, and milk enzymes (Figure 3B, curves b, d, and f, respectively). Maximum deviations in individual simulations were for g values, 0.0003; for $A(^1H)_1$, 0.01; and for $A(^1H)_2$, 0.03. Line widths were similar in the three simulations except that in Figure 3B, curve b, they were increased by a factor of 1.6 in the g_1 and g_3 orientations. [d] Parameters given are those used in the simulation of Figure 4b. [e] Values quoted are the average (±SD) of six data sets from the literature, on five different eukaryotic molybdenum-containing hydroxylases, as follows: milk xanthine oxidase (Hille et al., 1985; Lowe et al., 1972), rabbit aldehyde oxidase (Barber et al., 1982), turkey xanthine dehydrogenase (Barber et al., 1976), chicken xanthine dehydrogenase (Barber et al., 1980), and *Aspergillus nidulans* purine hydroxylase II (Coughlan et al., 1984). Note that in some cases saturating microwave powers were used, no doubt resulting in some spectral distortions and in slight shifts of apparent g values.

nation sphere of molybdenum in the functional and desulfo enzyme forms (Bray, 1988). On suitable reduction, the former gives an EPR signal named "Rapid" and the latter, one named "Slow". Despite the small quantities of the enzyme available, we succeeded in generating both signals and recording them at adequate signal-to-noise ratios, for samples both of the wild-type enzyme and of the mutant variant.

Spectra for the Rapid signal, generated with 1-methylxanthine for the two *Drosophila* enzyme variants and for milk xanthine oxidase, are illustrated in Figure 3A along with computer-simulated spectra. Corresponding data for the Slow signal are shown in Figure 3B and EPR parameters are summarized in Table V, together with data from the literature for comparison. The three Rapid spectra (Figure 3A) are indistinguishable from one another, either from visual inspection or from the results of iterative computer simulations (Table V). The same is true for the Slow signal (Figure 3B), with the exception that some broadening of the g_1 and g_3 features was observed in the spectrum from the *Drosophila* wild-type enzyme [see Figure 3B, curves a and b, and Table V, footnote *c*]. The Slow signal seems particularly susceptible to such broadening (Gutteridge et al., 1978b). The origin of this phenomenon is obscure, both in general and in the present work, though according to the above workers it can be brought

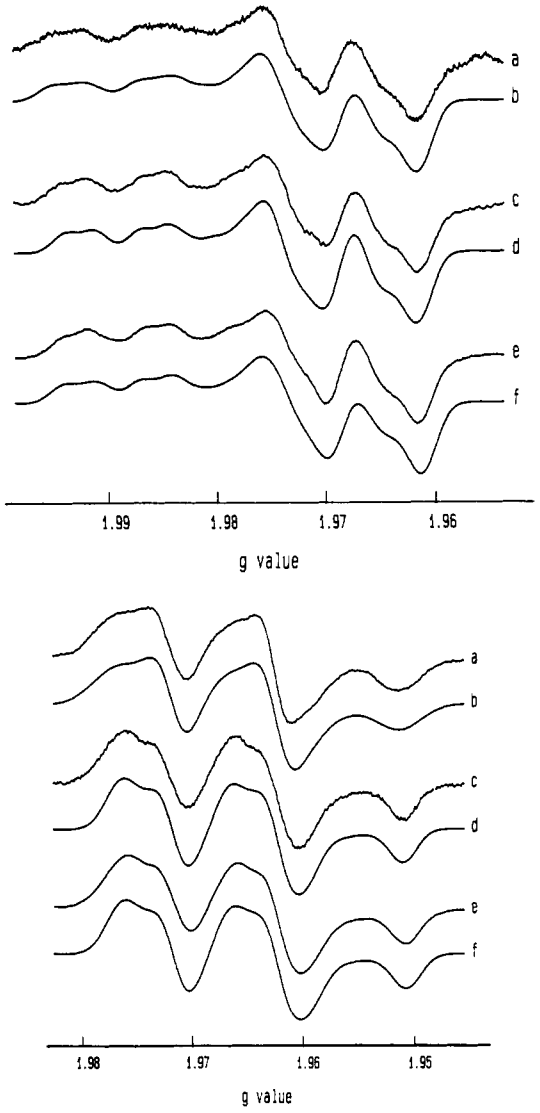

FIGURE 3: Molybdenum(V) EPR signals from wild-type and mutant *Drosophila* xanthine dehydrogenase, in comparison with those from milk xanthine oxidase, showing (A, top) the Rapid and (B, bottom) the Slow signal. (a and b) Wild-type enzyme (20 μM). (c and d) E89→K mutant (20 μM). (e and f) Milk xanthine oxidase (200 μM, 53% and 0% functional for panels A and B, respectively). Curves a, c, and e are experimental spectra and curves b, d, and f, respectively, computer simulations of these. Simulations were based on iterative fitting to individual spectra, but since the final parameters differed little, only averaged values and maximum deviations are listed in Table V (see footnote *c* in relation to line width differences in panel B, curve b). The Rapid signal was generated with 1-methylxanthine (2 mM; reaction time, 1–2 min) and the Slow with $Na_2S_2O_4$ (10 mol/mol of enzyme; 15-min reaction time). The buffer was 50 mM sodium Bicine, pH 8.2. Recording conditions were temperature, 120 K; microwave power, 5 mW; modulation amplitude, 0.29 mT; microwave frequency, 9.4 GHz.

about, e.g., merely by a change of buffer without a change of pH.

Overall, we conclude that the nature of all the atoms ligated to molybdenum, as well as the coordination geometry of the metal site, is identical in milk xanthine oxidase and the wild-type and E89→K mutant *Drosophila* xanthine dehydrogenases, with this being true for the enzymes in both the functional and the desulfo forms. This is the first time [cf. Wootton et al. (1991)] that identity between the molybdenum centers of molybdenum-containing hydroxylases from different species has been demonstrated to the level of precision achieved in the present work (cf. Table V).

Absolute intensities of the Rapid and Slow signals can provide information on the relative amounts of the functional and desulfo enzyme forms. Data on the *Drosophila* enzyme samples are summarized in Table VI. Conditions used for developing the signals were not rigorously standardized, and we did not optimize signal development, so no more than rough quantitation of the enzyme forms is possible. That the yield (17%) of the Slow signal from pure desulfo xanthine oxidase was comparable to that (12% and 19%, respectively) in our preparations of the two *Drosophila* enzyme variants at once confirms the earlier conclusion that in both of these most of the enzyme is in the desulfo form. The yield of Rapid signal from xanthine oxidase was 15% (Table VI). Extrapolation from the sample used to 100% functional enzyme would bring this value to 28%. This contrasts with the 4% yield of Rapid signal from our sample of the wild-type *Drosophila* enzyme. Simple proportion therefore suggests that $^4/_{28}$, i.e., 14% of the enzyme in this sample, is in the functional form, a value in satisfactory agreement with estimates for the content of functional enzyme given earlier. For the E89→K mutant, the yield of Rapid signal was more than twice that in the wild-type enzyme (Table VI). Accepting earlier conclusions that our samples of the two *Drosophila* enzyme variants contain similar proportions of functional enzymes, the high intensity of the Rapid signal may be explicable [cf. Hughes et al. (1992)] in terms of the absence (see below) of iron–sulfur centers from the mutant enzyme form, with this leading to an altered distribution of reducing equivalents in the reduced enzyme.

Table VI: Extent of Conversion to the Different EPR Signal-Giving Species for Wild-Type and Mutant *Drosophila* Xanthine Dehydrogenases: Comparison with Milk Xanthine Oxidase[a]

EPR signal	fractional conversion to signal (mol of e^-/mol of enzyme) wild-type enzyme	E89→K enzyme	milk enzyme
Rapid	0.04	0.09	0.15[b]
Slow	0.12	0.19	0.17[c]
Fe–SI + Fe–SII	1.9	0	2.2

[a] See Materials and Methods for details of procedures. [b] The sample used contained 53% of functional enzyme. [c] Desulfo enzyme was used.

Confirmation that both the wild-type and the mutant xanthine dehydrogenase preparations contain very large quantities of the desulfo enzymes raises the question of whether this form is present in vivo or whether it arises from degradation of the functional enzymes during the isolation procedures. In view of the stability of the isolated enzymes (Table II) and of the fact that no abnormal losses occurred during the isolation (Table I), we suggest that the desulfo forms are highly likely to be present in vivo.

EPR Spectra of Reduced Iron–Sulfur Centers in the Wild-Type Drosophila Enzyme. The EPR spectrum of the reduced wild-type *Drosophila* enzyme is shown in Figure 4, along with that of milk xanthine oxidase. Simulated spectra are also shown. Under the conditions of temperature and microwave power used, signals from both the Fe–SI and Fe–SII centers of the milk enzyme are expected (Bray, 1975) to be fully developed and not saturated. Thus, the spectrum will be the sum of those of the two species, present in equal amounts. The observed intensity for the *Drosophila* enzyme of close to 2 mol of unpaired electrons/mol of enzyme (Table VI) is thus in accordance with expectations. The line shape for the wild-type enzyme (Figure 4a,b) is entirely analogous to that of the milk enzymes (Figure 4c,d). This is confirmed by comparison of the parameters (Table V) used for the simulation (Figure 4b) with values from the literature. Perhaps the most reliable data on iron–sulfur signals from a molyb-

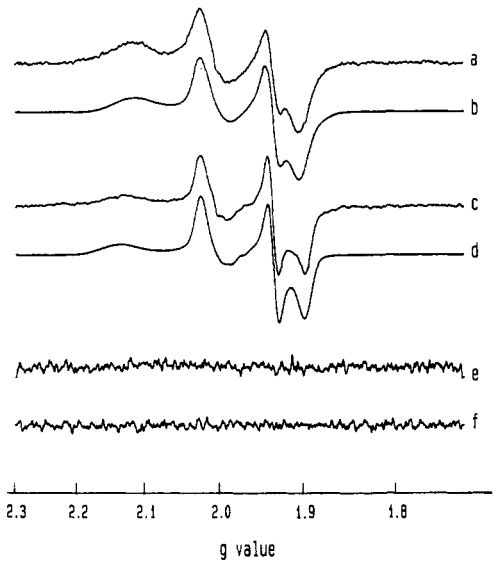

FIGURE 4: EPR signals from reduced iron–sulfur centers in *Drosophila* xanthine dehydrogenase, in comparison with those from milk xanthine oxidase. (a and b) Wild-type *Drosophila* enzyme (20 μM). (c and d) Milk xanthine oxidase (200 μM). Curves a and c are experimental spectra and curves b and d computer simulations, based on equimolar quantities of the two centers, Fe–SI and Fe–SII. Parameters used in curve b are given in Table V. Curves e and f show the absence of detectable signals from the E89→K mutant enzyme (20 μM) and are plotted at 4 times the amplification shown in curve a. In all cases, enzyme samples in 50 mM sodium Bicine buffer were reduced for 15 min with $Na_2S_2O_4$ (100 mol/mol of enzyme). Recording conditions in curves a, c, and e were temperature, 22 K; microwave power, 1 mW; modulation amplitude, 0.7 mT; microwave frequency, 9.4 GHz. In curve f, the temperature was 15 K, and the microwave power 10 mW.

denum-containing hydroxylase are those on milk xanthine oxidase of Hille et al. (1985). EPR parameters have been published for iron–sulfur signals from five different eukaryotic molybdenum-containing hydroxylases (see Table V, footnote *e*, for references). Values of individual workers vary somewhat, but it is clear from Table V that the two iron–sulfur centers of the wild-type *Drosophila* enzyme are entirely comparable to those of other eukaryotic molybdenum-containing hydroxylases.

The Absence of Iron–Sulfur Centers from the E89→K Drosophila Mutant Enzyme. We were quite unable to detect iron–sulfur EPR signals from our preparations of the E89→K mutant enzyme, under conditions similar to those used for the wild-type enzyme. The absence of such signals is demonstrated in Figure 4e (plotted with the vertical scale expanded 4-fold in comparison with Figure 4a). Lowering the temperature and increasing the microwave power in an effort to show up weak signals failed to demonstrate their presence (Figure 4f). Comparison of Figure 4, curves a and f, indicates that a reduced iron–sulfur center in the mutant, similar to Fe–SI of the wild-type enzyme and present in only 2–3% of the enzyme molecules, would have been detectable.

The absence of iron–sulfur EPR signals from the mutant enzyme, together with the form of the UV/visible absorption spectrum and the specific absorption data (Figure 2 and Table III), provides convincing evidence for the complete absence of the iron–sulfur centers from the mutant enzyme. In view of the ubiquitous presence of iron as a contaminant and of the small amounts of enzyme available, we did not attempt to perform chemical analysis for iron.

For milk xanthine oxidase, about two-thirds of the absorption at 450 nm is contributed by the iron–sulfur centers and one-third by the flavin (Komai et al., 1969). On reduction by dithionite, considerable absorption, both at 450 nm and at higher wavelengths, remains (Bray, 1975). The spectrum of the wild-type *Drosophila* enzyme changed similarly on reduction by dithionite (data not shown). The extent of bleaching at 450 nm that we observed was 65% (Table IV), close to the value of 69% for xanthine oxidase. In contrast, the FAD-like spectrum (Figure 2, curve d) of the E89→K mutant enzyme was essentially fully bleached by dithionite (Figure 2, curve f; Table IV). Also, the molar extinction coefficient of the mutant enzyme at 450 nm of 10.8 mM^{-1} cm^{-1} (Table III) is the same, within experimental error, as that of free FAD (11.3 mM^{-1} cm^{-1}), in agreement with the absence of iron–sulfur centers. The value of $A^{1\%}_{1cm}$ (280 nm) of 8.8 for the mutant enzyme is lower than the corresponding values for the wild-type enzyme and for milk xanthine oxidase. This again is consistent with the absence of iron–sulfur centers, since iron–sulfur model compounds [e.g., Wong et al. (1978)] absorb even more strongly in the region of 280 nm than they do at 450 nm.

The E89→K mutation involves the replacement of a negatively charged glutamate residue by a positively charged lysine, in the region of the peptide chain presumed (Wootton et al., 1991; Hughes et al., 1992) to lie between the two [2Fe–2S] clusters of the xanthine dehydrogenase subunit. For the mutation to result, as it does, in the complete deletion of both of the clusters, there must have been a profound change in the folding. This must have occurred despite the well-known stability of iron–sulfur clusters [see, e.g., Martin et al. (1990)]. Constituent with a major reorganization of the conformation of the molecule in the mutant are the differing SDS gel electrophoresis patterns (Figure 1C) observed for the mutant and wild-type enzymes, suggesting a change in the mutant in the sensitivity to proteolytic cleavage. However, significant differences between the mutant and wild-type enzymes in the region of the mutation would not be predicted on the basis of hydrophobicity (Hopp & Woods, 1981; Kyte & Doolittle, 1982; Hopp, 1986) or by using Garnier–Robson (Garnier et al., 1978) and Chou and Fassman (Chou & Fassman, 1974) structural predictions.

Measurements by Stopped-Flow of the Rate of Reduction of the Enzymes. Having established the absence of iron–sulfur centers in the mutant enzyme, which nevertheless has activity indistinguishable (Table II) from that of the wild-type enzyme in the xanthine (or pterin):NAD^+ oxidoreductase assay, we examined the effect of their absence on the catalytic properties of the enzyme. K_m values appeared[4] to be unaffected by the mutation. We therefore sought to obtain information on the rates of individual steps in the catalytic cycles of the two enzyme variants. For milk xanthine oxidase, reduction of the molybdenum center by xanthine is the rate-limiting step in enzyme turnover, with very rapid intramolecular electron transfer from molybdenum to the flavin and iron–sulfur centers, followed by relatively fast reoxidation via the flavin center (Olson et al., 1974). Measurements of the rate constant for anaerobic bleaching at 450 nm of the functional enzyme in stopped-flow experiments yielded a single first-order rate constant of 15.8 s^{-1} at 25 °C (Massey et al., 1969). This is despite the overall complexity of the processes, which involve (Olson et al., 1974) reduction not only of the flavin but also of the two iron–sulfur centers, with reduction brought about,

[4] In measurements on gel-filtered extracts [cf. Hughes et al. (1992)] in the pterin:NAD^+ oxidoreductase assay, for both the wild-type enzyme and the E89→K mutant, we found the following approximate apparent K_m values: K_m (pterin) 5 μM (at 330 μM NAD^+), K_m (NAD^+) 80 μM (at 11 μM pterin).

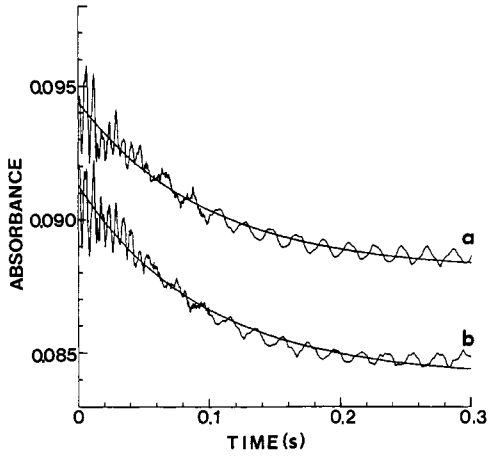

FIGURE 5: Time course of anaerobic bleaching by xanthine of wild-type and mutant *Drosophila* xanthine dehydrogenase, monitored at 450 nm and followed by stopped-flow. (a) Wild-type enzyme (4 μM, final concentration). (b) E89→K mutant (12 μM). The xanthine concentration was 100 μM. The experiment was carried out in 50 mM sodium Bicine, pH 8.2, at 23.5 °C. The solid lines through the experimental curves are least-squares fits to single exponentials.

not by one, but by three successive xanthine molecules.

We carried out stopped-flow measurements (Figure 5) to determine the rates of anaerobic reduction of the wild-type and E89→K mutant xanthine dehydrogenases by xanthine, and results are summarized in Table IV. Parallel experiments with milk xanthine oxidase yielded a rate constant of 12.5 s^{-1} at 23.5 °C, in agreement with the literature. The wild-type and mutant xanthine dehydrogenases gave rate constants of 10.2 and 10.6 s^{-1}, respectively, values that are within experimental error indistinguishable from one another and from that for the milk enzyme. A limiting value of the specific activity for the *Drosophila* enzyme variants of 5.0 μmol of substrate (mg of protein)$^{-1}$ min^{-1}, as suggested above, corresponds to k_{cat} = 12.2 s^{-1}. Thus, despite the absence from the mutant enzyme of the iron–sulfur centers, and the consequent difference in the precise processes being followed in the measurements on the two enzyme variants, the rate constants for reduction of the enzymes are the same and, as for xanthine oxidase, reduction is rate limiting in turnover.

Role of the Iron–Sulfur Centers. Sites of interaction of different oxidizing and reducing substrates with *Drosophila* xanthine dehydrogenase are discussed elsewhere in relation to intramolecular electron transfer pathways in the enzyme (Hughes et al., 1992). The present data make it abundantly clear, however, that reducing equivalents transferred to the enzyme from xanthine do not obligatorily pass through the iron–sulfur centers in their passage from molybdenum to flavin. Indeed, the inescapable conclusion is that the iron–sulfur centers of wild-type *Drosophila* xanthine dehydrogenase are irrelevant[5] to the normal turnover by the enzyme of xanthine or pterin in the presence of NAD^+ or of oxygen. Bearing in mind the very close relationships that exist among eukaryotic molybdenum-containing hydroxylases, it is highly likely that this conclusion applies also to other members of this group of enzymes, with the iron–sulfur centers not required for normal turnover. Whether, by extrapolation from our results, doubts should be cast on the essentialness of any other iron–sulfur centers in the functioning of enzymes of other types is quite uncertain. Assigning specific roles to iron–sulfur centers in enzymes is particularly difficult. Site-directed mutagenesis is currently being used to modify iron–sulfur clusters and so to probe their roles, e.g., in fumarate reductase (Werth et al., 1990). Work (Johnson et al., 1988; Manodori et al., 1991) on the latter enzyme includes the only example of which we are aware, apart from the present one, involving the genetic deletion of entire clusters.

The story so far is not complete, however, since the E89→K enzyme is from the *ry*^{5231} *Drosophila* strain, which has the *rosy* phenotype. Thus, at least in regard to the enzyme's influence on eye color, xanthine dehydrogenase function must be impaired in this strain in vivo. The most obvious conclusion from our work with artificial electron acceptors (Table II) is that phenazine methosulfate reacts with the iron–sulfur centers of the enzyme, with the result that, in the E89→K enzyme variant, turnover is blocked with this acceptor. We suggest that, in the as yet poorly understood reactions involved [cf. Reaume et al. (1990)] in eye color control in *Drosophila*, the enzyme employs, not NAD^+ or oxygen, but some unknown electron acceptor analogous to phenazine methosulfate and reacting with the enzyme, not at the flavin, but at the iron–sulfur centers.

[5] A referee has suggested that the iron–sulfur centers might perform some essential function in the wild-type enzyme which, e.g., because of a conformation difference, was no longer essential in the mutant. While we accept that this is possible, we prefer the simpler explanation that the iron–sulfur centers are redundant for normal turnover.

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